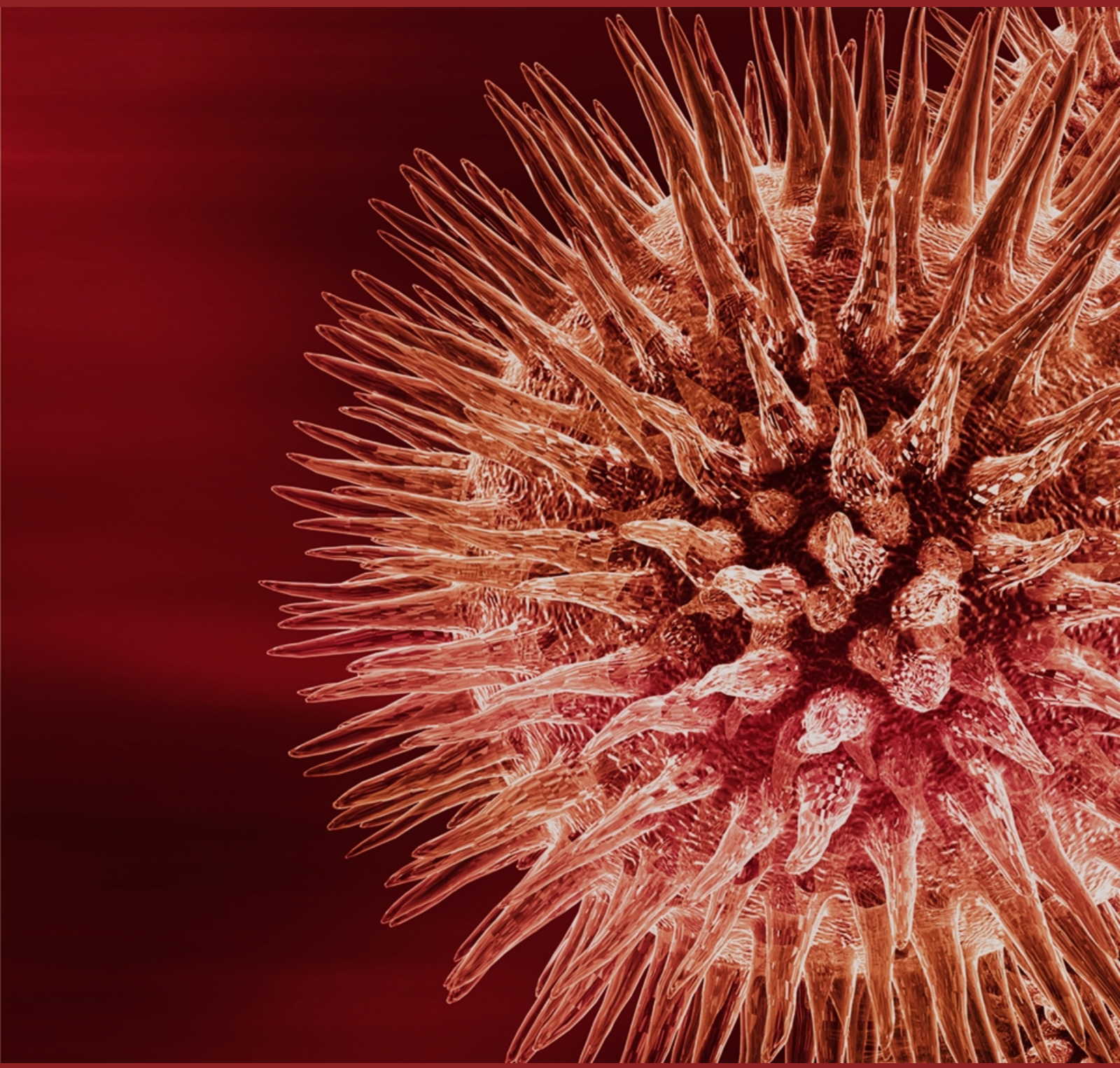
Journal of Biomedicine and Biotechnology

RNA Interference

Guest Editors: Mouldy Sioud and Abdelali Haoudi



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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 89018, Pages 1–2
DOI 10.1155/JBB/2006/89018

Editorial
RNA Interference

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Received 2 July 2006; Accepted 2 July 2006

The past decade has witnessed a true revolution in our understanding of how RNA can act as regulator of gene functions. Central to these new advances is a growing appreciation that small interfering RNAs (siRNAs) can induce sequence-specific destruction of homologous mRNAs in mammalian cells via a natural process referred as RNA interference (RNAi). During a period of only five years, RNAi has grown from a biological phenomenon to one of the most widely used tools in research. In effort to facilitate functional genomics with RNAi, several libraries of siRNAs or short hairpin RNAs have been constructed and screened in vitro and in vivo. Although RNAi has many advantages over other methods such as antisense and ribozyme technologies, the specificity of silencing is not absolute and there is a danger of "off-target effects," and activation of the innate immunity. Notably, the success of siRNAs as therapeutic agents largely depends on the development of a delivery vehicle that can efficiently deliver them to specific tissues or cells. A deeper understanding of the mechanisms of RNAi should allow better design of siRNA agents. The purpose of this issue is to review this exciting field and to provide the reader with current design rules, delivery strategies, and methods to minimize unintended siRNA effects.

It should be noted that the emergence of RNAi has helped to clarify another enigma of noncoding temporal RNAs or microRNAs (miRNAs). These tiny RNA regulators are being implicated in diverse biological pathways, ranging from development to neuronal differentiation and insulin production. In addition to their roles in cell biology, recent studies have implicated miRNAs in tumorogenesis and metastasis. Indeed, gene profiling analysis found a number of miRNAs that were upregulated in various cancers, which suggests a potential diagnostic and prognostic value. Also, the identification of virus-encoded miRNAs indicate that some viruses are able of exploiting RNA silencing as a convenient method for gene regulation of host and viral genes. Although we have learned much about the general mechanism underlying miRNA biogenesis, a detailed understanding of how miRNAs and related small RNAs work remains to be elucidated. This issue on RNAi also highlights the recent advances in understanding the biogenesis and expression of miRNAs in mammalian cells.

As our understanding of the functions of small RNAs and the mechanisms by which RNA activate innate immunity continues to increase, we should become better equipped to translate this naturally occurring process into our own therapeutic benefit.

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Mouldy Sioud received his DEA degree in pharmaceutical sciences from the University René Descates Paris V and his PhD degree in molecular biology from the University of Paris VII, France. He performed his postdoctoral fellowship at the Public Health Research Institute, New York, USA, in the laboratory of Karl Drlica. In 1990, he joined Prof Jacob Natvig's Group at the National Hospital, Institute of Immunology, University of Oslo, where he obtained in 1996 a second PhD degree in biotechnology and medicine. Presently, he is Group Leader and Professor in molecular immunology. His current research interests are in the area of RNA interference, innate immunity, and tumor immunology. He published more than 100 publications, edited three books (*Methods in Molecular Biology*), and he has received awards from both academic and industrial sources.

Abdelali Haoudi received his PhD degree in cellular and molecular genetics jointly from Pierre & Marie Curie University and Orsay University in Paris, France. He then joined the National Institutes of Health (NIEHS, NIH) for a period of four years after winning the competitive and prestigious NIH Fogarty International Award. He then joined the Myles Thaler Center for AIDS and Human Retroviruses at the University of Virginia Medical School, Charlottesville, then shortly after joining the faculty in the Department of Microbiology and Molecular Cell Biology at Eastern Virginia Medical School in Norfolk, Va, in 2001. He is interested in uncovering mechanisms by which mobile genetic retroelements, both retroviruses and retrotransposons, induce genetic instability and apoptosis in human cells and the molecular basis of cancer including cell cycle checkpoints and DNA repair mechanisms. He is also the Codirector of the Cancer Biology and Virology Focal Group. He has founded the *Journal of Biomedicine and Biotechnology* (http://www.j-biomed-biotech.org) and is also the Founder and President of the International Council of Biomedicine and Biotechnology (http://www.i-council-biomed-biotech.org).

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 65052, Pages 1–8
DOI 10.1155/JBB/2006/65052

Review Article

Essential Notes Regarding the Design of Functional siRNAs for Efficient Mammalian RNAi

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Received 25 January 2006; Accepted 17 April 2006

Short interfering RNAs (siRNAs) are widely used to bring about RNA interference (RNAi) in mammalian cells. Numerous siRNAs may be designed for any target gene though most of which would be incapable of efficiently inducing mammalian RNAi. Certain highly functional siRNAs designed for knockout of a particular gene may render unrelated endogenous genes nonfunctional. These major bottlenecks should be properly eliminated when RNAi technologies are employed for any experiment in mammalian functional genomics. This paper thus presents essential notes and findings regarding the proper choice of siRNA-sequence selection algorithms and web-based online software systems.

INTRODUCTION

RNA interference (RNAi) is the process of nucleotide-sequence-specific post-transcriptional gene silencing [1–5]. In the case of lower eukaryotes such as *Drosophila* and *Caenorhabditis elegans,* long dsRNA may be used as an RNAi inducer [6–15], while, possibly owing to interferon response [16–20], short interfering RNA (siRNA), a Dicer digestion product of long dsRNA, is widely used for knocking down mammalian genes through RNAi [21–23]. Interferon response can be brought about even by siRNA transfection [24–28] and may be permitted in most cultured cell experiments, in which siRNA concentration is equal to or less than 100 nM [29]. In therapeutic application, low siRNA-dependent interferon response would be a matter of concern [17, 18].

Theoretically, (n-20) siRNAs targeting for a gene n bp in length can be designed. In *Drosophila*, more than 90% of these siRNAs are capable of reducing target gene activity by more than 80% [29]. The design of siRNAs in the case of *Drosophila* as well as other lower eukaryotes would thus not involve any real difficulty. But about 80% of theoretically designable siRNAs would not be highly functional in the case of mammalian RNAi [29, 30]. With certain target genes rich in GC, nonfunctional siRNAs may increase by 95% or more of the total designable siRNAs [Y N et al, unpublished].

Mismatched siRNA may occasionally inactivate genes other than the target, an undesired side effect designated as the "off-target effect" [31, 32]. The molecular basis for this remains to be clarified [33] though mRNA cleavage, the climax of the RNAi reaction [34–38], requires a nearly strict nucleotide sequence identity between the mRNA target portion and sense strand (SS) of siRNA [33, 39]. Thus, at least some fraction of undesirable siRNAs, giving rise to the off-target effect through destabilization of mRNAs other than the target, may be eliminated by computer-based homology search [40–45].

In the design of highly functional siRNAs for mammalian RNAi, suitable sequence conditions or good algorithms for selection of highly functional siRNAs and good computer software suitable for genome-wide short-sequence homology search to minimize the off-target effect are indispensable.

Too many websites are available for functional siRNA search for mammalian RNAi as partly listed in Table 1. These websites may incorporate one or a few algorithms for functional siRNA selection previously determined based on biological validation data. Considerable mammalian RNAi data are presently available so that, in some websites, original algorithms may have been replaced with those modified to be more effective yet do not appear in scientific journals, thus making difficult the evaluation of individual website

Table 1: siRNA search websites.

Website	URL	Reference or company
BLOCK-iT RNAi Designer	https://rnaidesigner.invitrogen.com/	Invitrogen
DEQOR	http://cluster-1.mpi-cbg.de/Deqor/deqor.html	[46]
Gene specific siRNA selector	http://bioinfo.wistar.upenn.edu/siRNA/siRNA.htm	[47]
OptiRNAi	http://bioit.dbi.udel.edu/rnai/	[48]
RNAi Central	http://katahdin.cshl.org:9331/RNAi_web/	Hannon Lab
RNAi Design	http://www.idtdna.com/Scitools/SciTools.aspx	Integrated DNA Technologies
Sfold	http://sfold.wadsworth.org/	[49]
SiDE	http://side.bioinfo.ochoa.fib.es/	[50]
siDESIGN Center	http://www.dharmacon.com/sidesign/	Dharmacon Research, Inc
siDirect	http://design.RNAi.jp/	[40]
siRNA Design Software	http://www.cs.hku.hk/ sirna/	[51]
siRNA Design Tool	http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx	Qiagen
siRNA Selection Server	http://jura.wi.mit.edu/bioc/siRNA/	[52]
siRNA Sequence Selector	http://bioinfo.clontech.com/rnaidesigner/	Clontech
siRNA Target Designer	http://www.promega.com/siRNADesigner/	Promega
siRNA Target Finder	http://www.genscript.com/rnai.html	[53]
siRNA Target Finder	http://www.ambion.com/techlib/misc/siRNA_finder.html	Ambion
siRNA Wizard	http://www.sirnawizard.com/	InvivoGen
siSearch	http://sisearch.cgb.ki.se/	[42]
TROD	http://www.cellbio.unige.ch/RNAi.html	[54]

reliability. Consequently, the present study directs attention to basic frameworks and some related application problems of algorithms for the selection of highly functional siRNAs.

RNAi-INDUCING ACTIVITY AS AN INTRINSIC PROPERTY OF THE siRNA SEQUENCE

RNAi activity induced in mammalian cells is highly dependent on the particular sequence of siRNA used [29, 30] and may vary depending on transfected cell types or transfection efficiency. To examine these factors, various siRNAs targeting for the firefly luciferase gene (*luc*) were synthesized and transfected with *luc* encoding plasmid DNA into a variety of mammalian cell lines, which include human HeLa, HEK293, and colo205, Chinese hamster CHO-K1, and mouse E14TG2A ES cells [55]. The concentration of siRNA used in these experiments was 5–50 nM. siRNA-dependent RNAi activity was also examined in chicken embryos [29]. The transfection efficiency of colo205 is quite low and about 1/100 times as high as that of HeLa [55]. Neither difference in animal species from which cell lines or embryos were derived nor that in transfection efficiency had any significant effect on induced RNAi activity [29, 55]. RNAi activity induced in mammalian and chicken cells upon siRNA transfection may thus be determined primarily by the transfected siRNA sequences themselves as far as RNAi due to 10–50 nM siRNA is concerned.

THREE BASIC ALGORITHMS FOR SELECTING FUNCTIONAL siRNAs BASED ON BIOLOGICAL VALIDATION

Many experiments have been conducted to clarify possible sequence requirements of functional siRNAs for mammalian RNAi [29, 56–61]. Only three representative algorithms, which may be widely used for functional siRNA search for mammalian RNAi, are presented and discussed in the following.

Algorithm 1. This algorithm was developed by Ui-Tei et al [29]. As shown in Figure 1(a1), all siRNAs satisfying the following four sequence conditions are defined as class I siRNAs in Algorithm 1: (1) the 5′ antisense-strand (AS) end, A or U, (2) the 5′ SS end, G or C, (3) the 5′-terminal one-third of AS, A/U-rich, and (4) a long G/C stretch, absent from the 5′-terminal two thirds of SS. Validation data obtained using *luc* as a target indicated all of 40 class I siRNAs arbitrarily chosen to be capable of reducing target gene activity by more than 70% [29]. All RNAi experiments were conducted at 50 nM siRNA.

Algorithm 1 siRNAs with features completely the opposite to those of class I siRNAs except for condition (4) are defined as class III siRNAs (Figure 1(a2)). Validation indicated that all of 15 class III siRNAs arbitrarily chosen are incapable of inducing efficient mammalian RNAi [29]. Thus, most, if not all, class I siRNAs may possibly serve as siRNAs highly functional in mammalian cells. Class III siRNAs

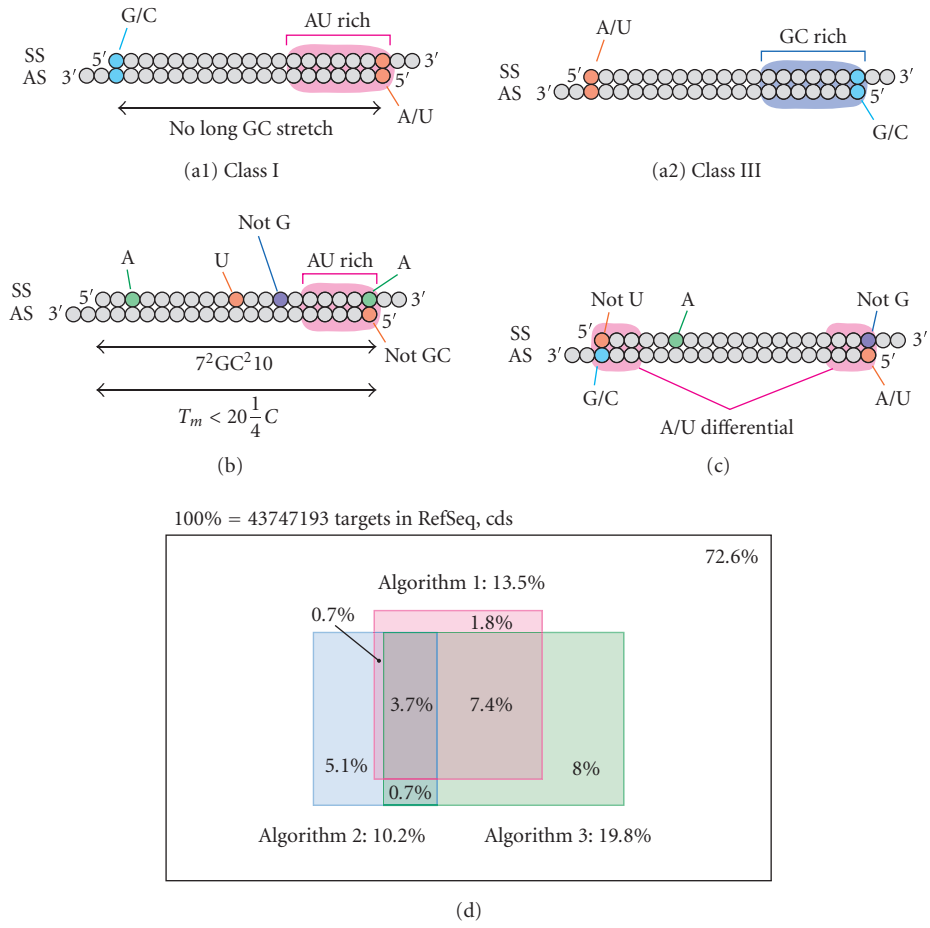

Figure 1: Three algorithms for siRNA design for functional RNAi in mammalian cells. (a) Algorithm 1. Highly functional class I siRNAs simultaneously satisfy the following four conditions: A/U at the 5′ AS end, G/C at the 5′ SS end, more than four A/U nucleotides in the 5′-terminal one-third of AS, and lacking a long G/C stretch in the 5′-terminal two-thirds of SS. Ineffective class III siRNAs possess features opposite to class I siRNAs. (b) Algorithm 2. There are 8 requirements for this algorithm: low G/C contents (30–52%), three or more A/U at the five 3′-terminal base pairs of SS, low internal stability lacking stable inverted repeats, and base preferences at SS positions 3, 10, 13, and 19. (c) Algorithm 3. A/U content in the 5′ AS end should be higher than that in the 5′ SS end. Base preferences are also required at positions indicated. (d) Difference in functional siRNA prediction between three Algorithms, 1, 2, and 3. 43747193 siRNA sequences were collected from human RefSeq sequences and classified using three algorithms.

are nearly incapable of inducing effective mammalian RNAi. With the *luc*, the total number of theoretically designable siRNAs is 1631 and class I siRNAs represent about 17%, which is roughly identical to the percentage (25%) of highly functional siRNAs estimated from validation data [29], class I siRNAs may thus constitute most, if not all, of siRNAs highly functional in mammalian RNAi.

Algorithm 2. This algorithm was proposed by Reynolds et al [59, Figure 1B] who carried out analysis of 180 siRNAs targeting mRNA of two genes and found the following characteristics associated with siRNA functionality: low G/C content, preference for low internal stability at the 3′-terminus of SS, and absence of inverted repeats. Furthermore, SS is presumed to preferably use A, U, and A at SS positions 3, 10, and 19, respectively. The 5′ AS terminal should not be G/C. G may not be present at position 13 (Figure 1(b)). In more than half of class I siRNAs, there are no base preferences at position 3 and 10 [29, 55], so that Algorithms 1 and 2, respectively, may predict considerably different siRNA sets to be functional.

Algorithm 3. This algorithm was proposed by Amarzguioui and Prydz [60] who carried out statistical analysis on 46 siRNAs and found Algorithm 3 to require the following features for functional siRNAs. The 5′ AS terminus and its SS partner are A/U and the 5′ SS terminus and its AS partner, G/C. An opposite combination of terminal bases may give rise to inadequate functionality. These authors also found that there is asymmetry in siRNA duplex end stability; that is, the A/U content differential for the three terminal nucleotides at both ends of the duplex may be considered essential to siRNA functionality. Furthermore, they noted A to prefer position 6 of functional siRNAs (Figure 1(c)), although only a small

fraction of class I siRNAs is associated with A at SS position 6 [29].

To examine in greater detail, relationships among the three algorithms, that the percentage of siRNAs considered functional by Algorithm 1 (class I) can be repredicted as functional by Algorithms 2 or 3 or vice versa, was determined (see [55, Figure 1D]. Based on the three algorithms, total possible siRNA sequences (4.4×10^7) designed using RefSeq human sequences (version 11) were found to be nonfunctional by as much as 73%. Class I siRNAs constituted 14% of the total theoretically predictable siRNAs, whereas Algorithms 2 and 3, respectively, predict 10 and 20% as functional siRNAs. Nearly 90% of class I siRNAs could be repredicted as functional by Algorithm 2 or 3 or both. Eighty four percent of siRNAs simultaneously predicted as functional by Algorithms 2 and 3 could be repredicted as functional or class I siRNAs by Algorithm 1. More than 50% of siRNAs predicted as functional by Algorithm 2 could not be predicted to be functional by Algorithm 3. Seventy seven percent of Algorithm 3 functional siRNAs could not be repredicted as functional by Algorithm 2. These findings may indicate that Algorithm 1 is capable of predicting the functionality of siRNAs more reliably than Algorithms 2 or 3.

ALIGNMENT ALGORITHM FOR SHORT NUCLEOTIDE SEQUENCES

Rapid homology comparison of the entire mRNA sequences with siRNA AS/SS sequences is indispensable for identifying off-target genes. BLAST [62] may not be a good software for making such comparison, since a number of off-target candidates are overlooked and too, considerable time is required for BLAST-based calculation. The Smith-Waterman local alignment algorithm [63] is accurate but time consuming to execute. Recently, Yamada and Morishita have developed a very rapid and accurate alignment algorithm for short nucleotide sequences [41] and this software can process 60 million siRNA sequences of 21 nucleotides in length in 10 hours when executed in parallel on ten inexpensive PCs. The hardware of Snøve Jr and Holen [64] provides similar performance although the number of processing units is not clearly specified. Websites using the Yamada-Morishita software or hardware of Snøve Jr and Holen should thus prove much more rapid and reliable compared to BLAST.

The base mismatch introduction studies indicate that transfected siRNAs occasionally cause phosphodiester-bond cleavage not only of the authentic mRNA target but also mutated targets with 1-2 base mismatches [33, 39]. But mutated targets with three or more mismatches may not undergo cleavage by transfection of the same siRNA [Y N et al, unpublished]. siRNAs less than 84 $(16/19 \times 100)$% homology in sequence to any part of total mRNAs other than the target should thus be used for RNAi, which would reduce the number of available functional siRNAs to 1/10 of the input. That is, only 10% of class I siRNAs or less than 2% of total siRNAs theoretically designable using human RefSeq sequences becomes available in mammalian RNAi when off-target effects due to mRNA destability are considered. Computational analysis indicated that, even so few available siRNAs, at least one functional class I siRNA can be assigned to more than 99% of human mRNA sequences (RefSeq sequences) [Y N et al, unpublished].

miRNAs involved in posttranscriptional gene silencing through translational regulation [65–73] possess less homology with the target, indicating siRNAs with lesser homology in some cases to possibly be involved in some off-target reactions [74]. The elimination of a large number of siRNA with low homology to mRNAs other than the target may render genome-wide gene silencing in mammalian cells quite difficult. The simultaneous use of a few to several siRNAs targeting for an identical gene (target gene) may possibly solve this problem since, in most cases, off-target targets would not be identical to each other [31, 32].

EXPERIMENTAL PARAMETERS POSSIBLY AFFECTING FUNCTIONALITY OF siRNAs

siRNA-mediated RNAi activity may vary significantly depending on not only the particular siRNA sequence but also parameters such as siRNA concentration, duration of siRNA exposure, and possibly target mRNA concentration and secondary structure within cells [29, 75]. Functional siRNAs in some cases have actually been found to induce maximum RNAi activity 1 day after transfection, whereas other siRNAs to express maximum activity on 2 or 3 days following transfection. Usually, functional-siRNA-dependent RNAi persists 1-2 weeks, whereas virtually no RNAi is induced within cells even after a long incubation with nonfunctional siRNAs. Class I siRNAs, capable of inducing highly functional RNAi when transfected at 50 nM, were considerably heterogeneous in capability of bringing about RNAi when used for 1-day transfection at the concentration of 50 pM (see [29] by Ui-Tei et al). Reduction in target gene activity varied from 20 to 60% depending on the sequences of class I siRNAs used. Thus, additional sequence conditions may possibly be found so as to define a subclass of class I with more functionality but in such a case, nearly complete genome-wide gene silencing might no longer be possible.

Recently, Kim et al [76] showed that a 27 bp long dsRNA with blunt ends is much greater in functionality than 21 bp long siRNA and suggested that short Dicer substrate dsRNA may be generally much more functional compared to authentic siRNAs 21 bp long. However, it was subsequently found that this is not a general feature of 27 bp long blunt-ended dsRNA [77]. In the absence of 3′ overhang, Dicer digests dsRNA uncontrollably, generating many products varying in length, most of which may not be as functional as 21 bp long highly functional siRNAs [77]. RNAi-inducing activity would thus appear to depend primarily on the presence of considerable highly functional siRNAs in the digestion products and so, consequently, 27 bp long blunt-end dsRNA would not be necessarily a good choice for highly efficient RNAi.

siRNA-OLIGOMER-DEPENDENT RNAi IN MAMMALIAN CELLS

Long dsRNA possessing 2-nucleotide 3′ overhangs at both ends is cleaved by Dicer from these ends to generate siRNAs having definite nucleotide sequences [28, 77–80]. Thus, should nearly all siRNAs produced by Dicer digestion belong to class I and the interferon response due to dsRNAs equivalent in length to siRNA oligomers not being significant, the induction of effective multiple-gene knockout in mammalian cells may occur with transfection of siRNA oligomers and this was recently found to be the case [28]. Through use of class-I-siRNA oligomers multiple-target gene knockout was clearly shown to take place.

DNA/shRNA-MEDIATED RNAi

RNAi can be induced by introducing DNA encoding both SS and AS of siRNA into mammalian cells. Both RNA polymerase III and II promoters, respectively, are used to express short hairpin RNA (shRNA) and longer RNA including shRNA sequence in the middle [81–90]. The primary transcript of RNA polymerase III is a mixture of shRNAs with two to several consecutive U's at its 3′ overhang [81–88]. Dicer cleavage sites of shRNAs vary depending on the length of 3′ overhangs [89] and accordingly, several different species of siRNAs are expected to be generated from shRNAs transcribed by polymerase III [88]. Thus, the presence of highly functional siRNAs in these Dicer digestion products is required for successful RNAi due to a polymerase-III-based system. In addition, four consecutive U's or A's should not be included in the nonoverhang sequences of AS and SS, respectively, since these sequences stimulate premature termination of polymerase-III-dependent transcription [88].

In polymerase II-driven expression systems, the primary transcript is long polyadenylated RNA (pri-miRNA-like RNA), which is recognized and cleaved by the nuclear microprocessor complex [91, 92]. This complex contains Drosha, an RNase III-type RNase that cleaves the pri-miRNA-like RNA to generate shRNA with a 2-nucleotide 3′ overhang [93]. The shRNA thus produced is converted mainly to two overlapping siRNAs through Dicer digestion (see [28]), indicating that successful RNAi requires the involvement of highly functional siRNAs in these siRNA products.

POSSIBLE MOLECULAR BASES OF ASYMMETRIC SEQUENCE REQUIREMENTS IN FUNCTIONAL siRNAs

Each mammalian Argonaute proteins (eIF2Cs) is comprised of a PRP motif and two domains: PAZ and PIWI [94]. Structural analysis of the Argonaute protein crystals from *Pyrococcus farious* indicated that the PIWI domain has essentially the same three-dimensional structure as ribonuclease *H* and that Argonaute may function as a slicer of mRNA [95]. PAZ and PIWI domains may recognize separately two ends of siRNA. The crystal structure of the PAZ domain from human Argonaute 1 suggested that the PAZ domain is anchored to the 2-nucleotide 3′ overhang of the siRNA duplex [96]. The PIWI domain from *Archaeoglobus fulgidus* contains a highly conserved metal-binding site that may recognize the 5′ nucleotide of AS of siRNA in a manner not dependent on sequence [97].

Algorithms 1 and 3 predict functional siRNAs to possess A/U and G/C at the 5′ AS and SS ends, respectively [29, 55, 60]. The GC pair is thermodynamically much more stable than the AU pair and thus, differences in stability in terminal base pair of the siRNA duplex may determine terminal sequence preference in highly functional and nonfunctional siRNAs, most probably by stimulating asymmetric binding of PIWI and PAZ domains to siRNA ends.

The 5′-terminal one-third of AS of functional class I siRNAs is A/U-rich, possibly due to preferable siRNA unwinding from its AS end [29, 56]. A one-step motor function of the putative siRNA helicase may unwind several base pairs from the A/U-rich siRNA end to stimulate formation of active RISC lacking SS of siRNA. Should this be the case, the introduction of base mismatches into the 3′-terminal third SS of siRNA may significantly increase the induced RNAi activity. Studies with *Drosophila* extracts showed a significant base-mismatch-dependent increase in RISC formation [56]. But, to date there are no data clearly confirm this in mammalian cultured cell experiments. Recently a part of RISC has been shown to be activated through cleavage of SS of siRNA at its center [98]. The presence of base mismatches in SS might be unfavorable to SS cleavage and this negative effect might partially prevent siRNA from being unwound.

ACKNOWLEDGMENTS

This work was partially supported by Special Coordination Fund for Promoting Science and Technology to K S, and Grants from the Ministries of Education, Culture, Sports, Science and Technology of Japan to K S and K U-T.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 83757, Pages 1–7
DOI 10.1155/JBB/2006/83757

Review Article

siRNA Efficiency: Structure or Sequence—That Is the Question

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Received 26 January 2006; Accepted 3 April 2006

The triumphant success of RNA interference (RNAi) in life sciences is based on its high potency to silence genes in a sequence-specific manner. Nevertheless, the first task for successful RNAi approaches is the identification of highly active small interfering RNAs (siRNAs). Early on, it has been found that the potency of siRNAs can vary drastically. Great progress was made when thermodynamic properties that influence siRNA activity were discovered. Design algorithms based on these parameters enhance the chance to generate potent siRNAs. Still, many siRNAs designed accordingly fail to silence their targeted gene, whereas others are highly efficient despite the fact that they do not fulfil the recommended criteria. Therefore, the accessibility of the siRNA-binding site on the target RNA has been investigated as an additional parameter which is important for RNAi-mediated silencing. These and other factors which are crucial for successful RNAi approaches will be discussed in the present review.

INTRODUCTION

RNA interference (RNAi) is a naturally occurring phenomenon of RNA-mediated gene silencing that is highly conserved among multicellular organisms (for recent reviews, see, eg, [1–4]). It is a post-transcriptional process initiated by double-stranded RNA molecules that induce degradation of a complementary target RNA. In the first step of the pathway, long double-stranded RNA molecules are chopped into shorter duplexes with 2 nucleotide overhangs at both 3′ ends by an endonuclease dubbed Dicer, the structure of which has been solved only recently [5]. The resulting 21 mer effector RNAs, named small or short interfering RNAs (siRNAs), are incorporated into a multimeric protein complex, the RNA-induced silencing complex (RISC). One of the two siRNA strands guides RISC to a complementary RNA. After hybridization the endonucleolytic "slicer" activity of RISC cleaves the target RNA, thus preventing its translation.

While long double-stranded RNA molecules can be employed to induce RNAi in lower eukaryotes, siRNAs being 21 nucleotides in length have to be used for gene silencing in mammalian cells in order to prevent the activation of an unspecific interferon response [6]. Due to the higher efficiency of siRNAs compared to traditional antisense oligonucleotides and ribozymes [7–9] and the relative ease of RNAi-mediated knockdown of target gene expression compared to knockout by homologous recombination, RNAi has rapidly become a standard technology in life sciences. Furthermore, siRNAs are not only new powerful research tools, but are also considered to be a promising new class of therapeutics [10–13].

In addition to siRNAs, endogenously expressed short double-stranded RNA molecules, referred to as microRNAs (miRNAs), entered the focus of current research (for a review, see [14]). These molecules are now believed to be important cellular gene regulators that play an important role in developmental processes and various diseases. At the beginning of the miRNA pathway, RNA polymerase II generates long primary RNAs that contain the miRNA sequences. These transcripts designated as pri-miRNAs are cleaved in the nucleus by an RNase III family enzyme, Drosha, to give the pre-miRNAs approximately 70–90 nucleotides with a 2 nucleotide 3′ overhang. After being exported to the cytoplasm, the pre-miRNA is recognized by Dicer and processed to generate the mature miRNA, which is incorporated into RISC. In contrast to siRNAs, however, miRNAs are capable of inhibiting translation of the targeted mRNA without degrading it (at least in mammalian cells). Still, the siRNA and miRNA pathways share many similarities. Elucidation of the mechanisms of miRNA activity therefore helps to understand the mode of action of siRNAs and vice versa.

Despite the great success of RNAi mediated approaches, the design of highly efficient siRNAs still remains a hurdle that has to be overcome. Initial expectations expressed on an antisense meeting in 2001 that there is no need to select for optimal siRNA target sequences [15] have soon been proven to be too optimistic, since a drastic variation of silencing efficiency was observed for different siRNAs directed against

the same target RNA [16]. It thus became clear that either factors intrinsic to the siRNA or properties of the targeted mRNA are crucial for the success of an RNAi approach. In the present review our current knowledge about factors that influence the potency of siRNAs will be summarized and advice will be given that helps with the generation of efficient molecules. It will, however, become obvious that we do not yet know all relevant features so that even the sophisticated design algorithms available to date do not guarantee satisfactory activity of the proposed siRNAs.

THERMODYNAMIC PROPERTIES OF EFFICIENT siRNAs

Early on, recommendations have been given for the selection of siRNA target sites [17]: the selected region should preferably be located in the coding region, at least 50 nucleotides downstream of the start codon; the GC-content should be approximately 50%; and a sequence motive AA N_{19} TT was suggested to be advantageous. A blast search is necessary to ensure that the siRNA has no significant homologies with other genes than the intended target. Even though these selection criteria have been employed with great success in numerous RNAi experiments, a further increased hit rate for highly potent siRNAs was desirable for the generation of large libraries. Significant progress towards the design of active siRNAs was achieved when an unexpected asymmetry concerning the incorporation of the two strands of siRNAs and miRNAs was found in two independent studies [18, 19]. Analysis of the known miRNA sequences in the context of miRNA precursor hairpins revealed a low stability of the 5′ end of the antisense strand compared to the 5′ end of the sense strand [18]. Subsequently, the same feature was observed for siRNAs. Functional duplexes displayed a lower relative thermodynamic stability at the 5′ end of the antisense strand than nonfunctional duplexes. The finding that the relative stabilities of the base pairs at the termini of the two siRNA strands that determine the degree to which each strand is fed into the RNAi pathway led to the hypothesis that strand incorporation into RISC is determined by an RNA helicase that initiates dissociation of the miRNA or siRNA duplex at the end with the lower thermodynamic stability [19].

These findings were further refined in a systematic analysis of 180 siRNAs targeting the mRNAs of two genes [20]. In addition to the relative stability of both ends of the siRNA, base preferences at certain positions of the duplex were identified in functional siRNAs. A set of eight criteria was used in an algorithm intended to improve the selection of potent siRNAs (Table 1 and Figure 1). A total of 6 or more points according to this scoring system was proposed to significantly increase the probability for efficient gene silencing.

Independent studies analysing the activities of siRNAs against different mRNAs confirmed the basic outcome of these studies [eg, [21, 22]]. Although some base preferences at certain positions of the siRNAs were either questioned or added to the list, the relative thermodynamic stability of the siRNA termini was verified to be a major determinant of the functionality of siRNAs. Somewhat different results were obtained, when a database was compiled consisting of 398 siRNAs against 92 genes from 30 different studies, in order to overcome a major shortcoming of earlier studies, the low number of genes being targeted [23]. Bioinformatic analysis of the data set led to a set of rules (termed "Stockholm rules") that differs from the scoring systems described above.

Table 1: Design criteria for siRNAs according to Reynolds et al [20].

Criterion	Score
GC content between 30–52% (in the 19 mer siRNA duplex)	1
A or U at positions 15–19	1 for each
A at position 19	1
A at position 3	1
U at position 10	1
G or C at position 19	−1
G at position 13	−1

Various academic groups and commercial vendors developed a software for designing siRNAs based on the identified features of active siRNAs. A list of freely available web tools is given in Table 2. Some additional prediction servers were introduced in a special web server issue of Nucleic Acids Research of July 2004.

In a more recent study, a set of approximately 2200 randomly selected siRNAs targeting 24 mRNA species was used to train a neuronal network to predict the activity of siRNAs [25]. Statistic analysis of the large data set revealed some of the criteria discovered previously, but also identified new motives that are overrepresented in potent siRNAs. The approach to train an artificial neuronal network goes beyond earlier efforts like the above-mentioned scoring system, which uses a linear summation of parameters, in that it can handle complex sequence motifs and synergistic relations between two or more parameters. The neuronal network-based algorithm was finally employed to design a library of approximately 50.000 siRNAs that cover the human genome with a redundancy of two siRNAs per gene.

Taken together, the analysis of the sequences of active and nonfunctional siRNAs clearly revealed that the two strands of an siRNA duplex are not equally eligible for assembly into RISC. Rather, the relative stability of both ends of the siRNA is widely considered to determine which of the strands will preferentially participate in the RNAi pathway. It is therefore advisable to take into account the proposed criteria for active siRNAs when designing siRNAs against a new target. It has to be mentioned, however, that following these algorithms does not guarantee for the success of an RNAi approach. On the contrary, numerous highly efficient siRNAs have been published that do not obey the rules. Before addressing further determinants of siRNA activity in more detail, a short summary of structural studies will be given that may account for the asymmetric incorporation of the two siRNA strands into RISC.

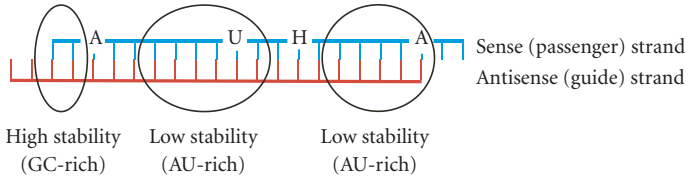

Figure 1: Features of efficient siRNAs according to Reynolds et al [20]. The relative stability of both ends of the siRNA as well as the bases in certain positions influences the activity of siRNAs (H = A, C or U).

Table 2: Web sites for the design of effective siRNAs (based on [24] with modifications).

Source	URL
Dharmacon	www.dharmacon.com/sidesign/
Hannon Lab	katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=siRNA
Integrated DNA Technologies	scitools.idtdna.com/RNAi
Sonnhammer Lab	sisearch.cgb.ki.se
Invitrogen	rnaidesigner.invitrogen.com/sirna
McManus Lab	
Qiagen	http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx
Sfold Algorithm	http://sfold.wadsworth.org/index.pl
Whitehead Institute	http://Jura.wi.mit.edu/siRNAext
MWG-Biotech	http://www.mwg-biotech.com/html/s_synthetic_acids/s_sirna_design.shtml

STRUCTURAL BASIS FOR STRAND ASYMMETRY

In recent years, significant progress has been made to elucidate the molecular basis of RNAi and to understand the asymmetric strand incorporation (for a review, see [26]). The catalytic activity of RISC, termed slicer, which leads to the cleavage of the target RNA, has been identified to be located in the Argonaute2 (Ago2) protein [27]. Ago2 contains two major domains referred to as PIWI and PAZ (acronym for PIWI/Argonaute/Zwille). Crystallographic analysis revealed the PIWI domain at the C-terminus of the protein to closely resemble the structure of RNase H [28]. This enzyme cleaves the RNA component of an RNA/DNA hybrid. The PIWI domain of Ago2 can thus be regarded as a variant of the RNase H structure motive specialized in cleavage of one strand of double-stranded RNAs.

Recombinant human Ago2 and an siRNA were found to form a minimal RISC that accurately cleaves substrate RNAs [29]. Interestingly, only single-stranded siRNA could be specifically incorporated into recombinant Ago2, whereas photoreactive double-stranded siRNA did not crosslink with Ago2. This finding indicates the importance of the RISC loading complex (RLC) for efficient incorporation of the siRNA into the Ago2 protein. In *Drosophila melanogaster*, a heterodimer consisting of Dicer-2 and the double-stranded RNA binding protein R2D2, which contains the siRNA, was found to be important for RISC assembly [30]. R2D2 binds the thermodynamically more stable end of the siRNA, that is, the 3′ end of the guide strand, and can thus determine which one of the strands will be associated with Ago2. It has therefore been described as the "protein sensor for siRNA thermodynamic asymmetry."

In human cells, the HIV-1 trans-activating response RNA-binding protein (TRBP) has been found to recruit the Dicer complex to Ago2 [34]. Based on these findings a model has been proposed for RISC assembly and function [31] that is depicted in Figure 2. In cytoplasm, RISC containing Dicer, TRBP, and Ago2 recognizes hairpin RNAs like pre-miRNAs. The RNase III Dicer generates ~22 nt long duplexes which remain associated with RISC as a ribonucleoprotein complex. In analogy to R2D2 from *Drosophila*, TRBP and Dicer are likely to sense the thermodynamic asymmetry between the two ends of the duplex. Two recent reports suggest that the passenger strand is cleaved, before being removed from the Ago2 protein [32, 33]. The guide strand remains bound to the active RISC and recognizes target RNAs by complementary base pairing. The PIWI domain of Ago2 cleaves the target RNA. After release of the cleavage products, RISC can undergo further rounds of target RNA destruction. Interestingly, none of these steps requires energy from ATP hydrolysis. Although RISC can utilize 21 mer siRNA duplexes, pre-miRNA-type Dicer substrates result in a 10-fold higher activity [31].

TARGET SITE ACCESSIBILITY

Although there is no doubt that the design criteria described above increase the success rate to generate active siRNAs, a survey of published RNAi experiments readily reveals that many siRNAs are highly potent although they do

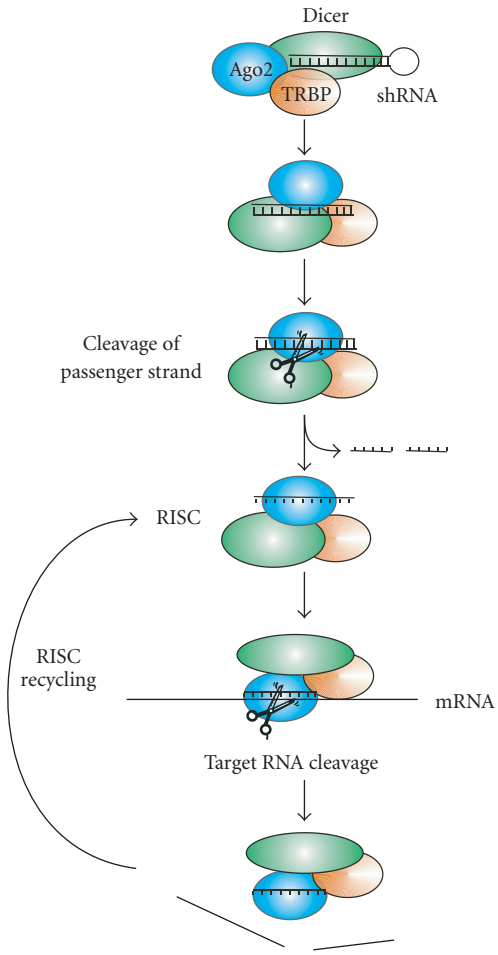

Figure 2: Model for assembly and function of RISC according to [31] under consideration of [32, 33].

not fulfil the recommendations. Even more intriguing is the fact that siRNAs may be unsuitable to silence their target although they comply with these rules. It is thus obvious that additional features have to be considered to optimize the efficiency of RNAi. Some earlier studies had already suggested that the structure of the target RNA may influence siRNA activity [35–37]. When it became clear that the design algorithms based solely on thermodynamic parameters of the siRNA are helpful tools, but do not guarantee success of RNAi approaches, target-site accessibility came back into the focus.

Luo and Chang [38] described the local mRNA structure at the target site as the main cause for the positional effect of different siRNAs. As a reliable parameter for target site accessibility, they introduced the "hydrogen bond index" representing the average number of hydrogen bonds formed between nucleotides in the target region and the rest of the mRNA. This index, which has to be determined by bioinformatic secondary structure prediction, was found to correlate inversely with the gene-silencing effect. Further experiments revealed that the tight stem-loop structure of the HIV-1 transactivation response element (TAR) is detrimental to silencing by RNAi [39]. In contrast, the location of the siRNA-binding site within a translated or noncoding region of the mRNA had only marginal effects.

A systematic global analysis was performed with a set of siRNAs directed against two target RNAs, for which the accessibility of the siRNA target sites was determined by an iterative computational approach and by experimental RNase H mapping [40]. IC_{50}-values as well as the maximal extent of target suppression were significantly improved for siRNAs against accessible local target sites compared to those siRNAs which targeted inaccessible regions of the mRNAs. In contrast, the relative thermodynamic stability of both ends of the siRNA was not found to be a suitable marker for siRNA activity. This finding was further strengthened by a kinetic analysis of isolated human RISC [41]. An siRNA directed against the highly structured RNA of the HIV-1 TAR was found to be incapable of target RNA cleavage. When the tight structure was disrupted by the addition of an oligonucleotide consisting of 2′-O-methyl RNA, target-site accessibility increased leading to enhanced cleavage of the TAR RNA.

In a recent study, we aimed at deciphering the contributions of both factors, that is, the thermodynamic properties of the siRNA and the target RNA structure, to the efficiency of an RNAi approach by constructing a set of intentionally designed target sites [42]. A highly active siRNA, which is capable of silencing its full-length target RNA in the subnanomolar range, maintained its potency when directed against the isolated target site fused to the green fluorescent protein (GFP). Interestingly, a fusion construct with the siRNA-binding site in reverse orientation was found to be silenced to a much lower extent, confirming the existence of a strand bias. However, incorporation of the original target site into a tight hairpin structure was detrimental to silencing as well. Further experimental and bioinformatic analysis of a set of target RNAs with varying degrees of target-site accessibility revealed a linear correlation between the local free energy in the siRNA-binding region and the extent of gene knockdown. These findings demonstrate that the thermodynamic properties of the siRNA itself as well as the structure of the target RNA both influence the efficiency of an siRNA. We therefore proposed a model, according to which the outcome of an RNAi approach is determined at two points of the multistep process (Figure 3). Firstly, asymmetric strand incorporation into RISC is controlled by thermodynamic properties of the siRNA; secondly, accessibility of the target site may further modulate the efficiency of silencing. Even siRNAs with favorable thermodynamic properties may thus be incapable of inhibiting gene expression in cases in which the binding region is inaccessible due to tight secondary structures.

Design of siRNAs according to the criteria recommended by Reynolds et al [20] frequently results in satisfactory inhibition of gene expression. Some targets, however, are refractory to RNAi-mediated silencing, most likely due to the existence of stable secondary structures. For example, we and others failed to identify efficient siRNAs against the highly structured 5′ untranslated region of plus-stranded RNA viruses and were more successful when targeting less tightly arranged parts of the coding region [43–47]. In some cases, it

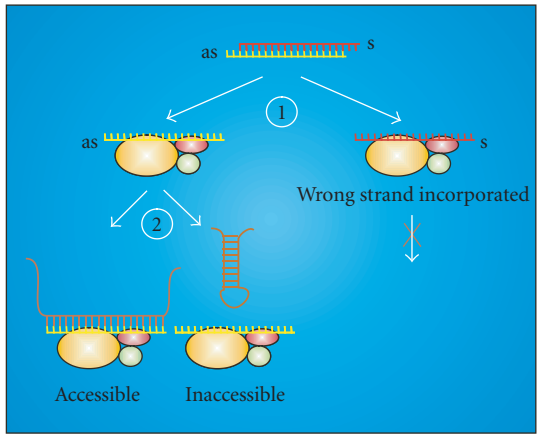

Figure 3: Efficiency of an siRNA is determined at two points of the RNAi pathway. (1) A strand bias exists that is defined by the intrinsic thermodynamic properties of the siRNA duplex, that is, by the relative stability of both ends. (2) A highly ordered structure may have a detrimental influence on the hybridisation of the siRNA/RISC to its target site and may therefore reduce the efficiency of the silencing process, even in cases in which the intended antisense strand is favored for incorporation into RISC. (Reprinted with slight modifications from the Journal of Molecular Biology; see [42], with kind permission from Elsevier.)

might be advisable to take the target RNA structure into account as well. Several freely available design algorithms, for example, the *S*fold web server (http://sfold.wadsworth.org [48]) and the siRNA design tool offered by MWG-biotech (http://www.mwg-biotech.com [49]) allow the design of siRNAs based on thermodynamic properties of the duplexes with consideration of the predicted secondary structure of the binding region of a potential siRNA.

STRATEGIES TO IMPROVE siRNA EFFICIENCY

Detailed bioinformatic analysis of the large set of sequence-activity relationships reported by Huesken et al [25] confirmed that the score according to Reynolds et al [20] as well as the target-site accessibility correlate with the extent of siRNA-mediated gene silencing. However, this investigation clearly revealed that both parameters are insufficient to fully explain or predict the potency of siRNAs (G. Schramm, personal communication). Thus, further factors can be expected to influence the functionality of siRNA molecules. Recently, Patzel et al [50] suggested that the structure of the guide strand could be another feature, which is crucial for the efficiency. Employing a series of siRNAs with different structures, guide strands that do not form defined structures or possess freely available terminal nucleotides, mainly at the 3′ end of the guide strand, were found to increase the efficiency of siRNAs (Figure 4). In contrast, structures with base-paired ends were virtually inactive. Interestingly, in this study neither the thermodynamic duplex profiles nor target mRNA structure were found to be of major importance for the silencing process.

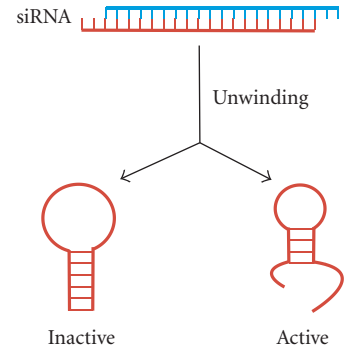

Figure 4: Influence of guide RNA structure on siRNA efficiency [50]. siRNA guide strands with base-paired termini were found to be inactive, whereas guide RNAs with freely accessible ends (mainly 3′ ends) were highly efficient.

A strategy to circumvent the need to identify suitable individual siRNAs is to use mixtures of siRNAs. To this end, long double-stranded RNA molecules have been processed in vitro by *Escherichia coli* RNase III [51]. The resulting pool of siRNAs, dubbed endoribonuclease-prepared siRNAs (esiRNA), can subsequently be transfected into cells to silence the corresponding gene. This efficient and cost effective method allowed the rapid generation of a large library consisting of more than 5000 esiRNAs [51]. It is still under debate whether this approach will elicit severe off-target effects due to the large number of sequences contained in the pool. It has, however, also been argued that pooling of siRNAs might decrease unspecific effects, since this strategy dilutes out the off-target effects of each individual siRNA, while retaining the total target-specific silencing capacity.

Two independent studies described additional approaches to enhance the efficiency of a single siRNA. Conventional siRNAs consist of a 19 mer double-stranded region and two nucleotide overhangs at the 3′ ends of each strand. Accordingly, short hairpin RNAs used for vector expression are designed with a 19 mer duplex, a loop connecting both strands, and two to four uridines at the 3′ end of the antisense strand. The two more recent publications now report that longer siRNA duplexes are up to 100-fold more potent than the corresponding conventional 21 mer siRNAs [52, 53]. In one of these studies a set of chemically synthesised siRNAs of varying length was used [52]. The optimum of silencing efficiency was found for siRNAs being 27 nucleotides in length. These 27 mers were even suitable to target sites that are refractory to silencing by 21 mer siRNAs. Importantly, the 27 mer duplexes did not activate the interferon response or protein kinase R. The authors of the second publication found 29 mer short hairpin RNAs to be particularly potent inducers of RNAi [53]. The higher efficiency of longer double-stranded RNA duplexes might be due to the fact that these siRNAs and shRNAs, respectively, are initially processed by Dicer to give 21 mers. As described above, mechanistic models based on copurification experiments [31] indicate that Dicer is involved in the loading process of siRNAs into RISC, thus explaining the improved potency of Dicer substrates compared to traditional 21 mer siRNAs. In a follow-up study, 27 mer

duplexes with 2-base 3′-overhangs were found to be superior compared to blunt-end duplexes [54]. Interestingly, asymmetric strand utilization was found with the strand carrying the overhang being preferred for silencing. The authors conclude that Dicer processing confers functional polarity within the RNAi pathway for longer double-stranded RNAs.

Recently developed strategies to generate siRNAs from a miRNA environment went along the same lines to employ Dicer substrates for silencing. Stegmeier et al [55] generated an siRNA by replacing a naturally occurring miRNA by a target-specific siRNA sequence flanked by ∼125 bases of 5′ and 3′ sequence derived from the primary miRNA transcript. This construct can be expressed from both Pol III and Pol II promotors, thus opening the road to use tissue-specific promotors. The microRNA-type expression of shRNAs has been found to be superior compared to conventionally expressed isolated shRNAs and has been used to generate large libraries covering a substantial fraction of the predicted genes in the human and mouse genomes [56].

SUMMARY

Various factors have been identified that contribute to the efficacy of small interfering RNAs. Thermodynamic properties of a given siRNA itself influence its asymmetric incorporation into the RNA-induced silencing complex. Furthermore, the local structure of the targeted RNA might render the siRNA-binding region inaccessible, thus preventing efficient silencing. Additional factors like the availability of free ends of the siRNA antisense strand have been described to be relevant to the induction of RNAi. It is, however, clear that all of these features still do not provide an exhaustive description of the determinants of siRNA potency. We can therefore expect additional factors to be identified that contribute to the activity of siRNAs. Additional research is needed to further increase the success rate when designing siRNAs against a new target RNA.

ACKNOWLEDGMENTS

I wish to thank Prof. Dr. Volker A. Erdmann for his continuous support and all the members of my research team for their excellent work. Furthermore, I want to thank Diana Rothe, Steffen Schubert, Thomas Röhl, Harry Kurreck, and Gerrit Schramm for critical reading of the manuscript. The author's work is funded by the Deutsche Forschungsgemeinschaft (SFB/TR19), by the Fonds der Chemischen Industrie, and by the RiNA Network for RNA Technologies which is funded by the city of Berlin, the Federal Ministry of Education and Research, and the European Regional Development Fund.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 23429, Pages 1–4
DOI 10.1155/JBB/2006/23429

Review Article

Molecular Basis for the Immunostimulatory Potency of Small Interfering RNAs

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Received 5 March 2006; Revised 15 June 2006; Accepted 28 June 2006

Small interfering RNAs (siRNAs) represent a new class of antigene agents, which has emerged as a powerful tool for functional genomics and might serve as a potent therapeutic approach. However, several studies have showed that they could trigger several bystander effects, including immune activation and inhibition of unintended target genes. Although activation of innate immunity by siRNAs might be beneficial for therapy in some instances, uncontrolled activation can be toxic, and is therefore a major challenging problem. Interestingly, replacement of uridines in siRNA sequences with their 2′-modified counterparts abrogated siRNA bystander effects. Here we highlight these important findings that are expected to facilitate the rational design of siRNAs that avoid the induction of bystander effects.

INTRODUCTION

A variety of well-documented approaches have been developed for inhibiting gene expression. These include the use of antisense RNAs, oligodeoxynucleotides (ODNs), ribozymes, and RNA interference (RNAi) [1–3]. Among these approaches, RNAi-mediated mRNA degradation is emerging as the most highly effective antigene strategies [2, 3]. Although the same phenomenon was described in transgenic plants in the late 1980s [4], only in 1998 Fire and colleagues have demonstrated that ds RNA introduced into *Caenorhabditis elegans* can silence the expression of homologous target gene by directing degradation of its mRNA [5]. During RNAi, the precursor double-stranded (ds) RNA is processed by the Dicer endonuclease into short 21–24 nucleotides ds siRNAs containing 2-base 3′-overhangs. Subsequently, the siRNA duplexes are then incorporated into a multiprotein complex, the RNA-induced silencing complex (RISC), which mediates the degradation of mRNAs with sequence homologous to the siRNA. Recent studies have showed that nucleotides 2–8 of the siRNA antisense strand form a seed sequence that directs target mRNA recognition [6]. Some of the protein components of RISC have been identified and assigned functions. Argonaute 2 (Ago2) is the RNA endonuclease that cleaves target mRNA [7].

Despite the demonstration of RNAi in plants and worms, researchers have been pessimistic about using RNAi in mammalian cells due to the induction of the interferon (INF) pathway by long ds RNAs (> 30 nucleotides), leading to nonspecific inhibition of protein synthesis and degradation of mRNAs [8]. However, Tuschl and colleagues found that siRNA duplexes mimicking the siRNAs generated by Dicer cleavage in lower organisms could trigger RNAi without inducing the IFN pathway, which is observed with long double-stranded RNA in most cell types [9]. This discovery has paved the way for the study of gene function in mammalian cells. In contrast to invertebrates, mammalian cells do not have transitive RNAi because of the absence of RNA-dependent RNA polymerase [2, 3]. Consequently, the number of siRNA molecules introduced into a cell limits gene silencing induced by synthetic siRNAs. To circumvent this potential problem, several groups have developed expression vectors that direct the synthesis of RNA duplexes or short hairpin RNAs (shRNAs) in mammalian cells, leading to sustained production of siRNAs [3]. One approach for silencing relies on the expression of shRNAs under the control of H1 or U6 pol III promoter, which is active in most mammalian cell types.

During the last 5 years, siRNA-mediated gene knockdown has become a standard method for studying gene function and drug target validation. However, despite the success that has been achieved, some serious concerns such as the activation of innate immunity, inhibition of unintended target mRNAs, and potential interference with regulatory functions

of endogenous microRNAs need to be resolved prior therapeutic applications in humans [2, 10–12].

CELLULAR RESPONSE TO siRNAs

The gradual maturation of RNAi technology from the laboratory to the clinic involves several major challenges, many of which still need to be resolved. These include delivery to target cells, intracellular stability, and specificity [2, 3]. It should be noted that the introduction of synthetic siRNAs into mammalian cells in culture is relatively simple by transfection reagents [9]. siRNAs can be chemically synthesized, or produced by in vitro transcription or by digestion of long dsRNAs by recombinant RNase III or Dicer [2, 3]. Most of the techniques that have been used for antisense delivery can be applied to synthetic siRNAs and to DNA constructs engineered to express shRNAs. These include electroporation, cell microinjection, and lipophilic transfection [13].

Although siRNAs were initially thought to be small enough to avoid the activation of the IFN pathway [9], recent studies showed they could activate innate immunity in mammalian cells [10, 14–16]. In this respect, Sledz and colleagues reported that siRNA could activate PKR, and the effects were sequence independent and do not occur with the sense or the antisense RNA used to prepare the siRNA duplexes [15]. In contrast, Kariko and colleagues found that siRNA could activate cytokine and interferon production via TLR3, a receptor known to bind viral ds RNAs [16]. TLR3 is mainly expressed on the cell surface. However, we and others recently have demonstrated that PKR and TLR-3 do not represent the major mechanism by which chemically synthesized siRNA activate innate immunity [17–19]. In addition, internalization of ds siRNAs or ss siRNAs is required for immune activation. Indeed, inhibitors of endosomal maturation/acidification like bafilomycin A1, a drug that inhibit endosomal H+-proton pumps, blocked immune activation [17], indicating the involvement of endosomal TLR7 and TLR8. Consistent with the role of endosomal TLRs in siRNA signaling, TLR7 knockout mice did not mount immune activation in response to siRNAs [18]. Taken together, the available data indicate that immune cells such as monocytes and dendritic cells recognize ds siRNAs and ss siRNAs through TLR7 and TLR8, leading to the production of inflammatory cytokines and type I interferons [20]. We also found that ss siRNAs are more effective than ds siRNA in triggering TLR7 and TLR8 responses [17, 21]. In addition, we have found that human bone marrow hematopoietic CD34+ progenitor cells express TLR7 and TLR8. Interestingly, incubation of these cells with either immunostimulatory synthetic siRNAs or R848, a specific ligand for TLR7 and TLR8, induced their differentiation into the myeloid lineage (Sioud et al in preparation). Thus, the interaction of viral RNAs with CD34+ progenitor cells may increase the pool of innate immune cells.

As mentioned above mammalian cells respond to siRNAs, provided they are delivered to the endosomes. Notably, the immune system uses a set of germ line encoded receptors called pattern recognition receptors (PRRs) to recognize common microbial structures known as pathogen associated molecular patterns [22]. Whereas several TLRs are expressed in the cell surface, TLR3, TLR7, TLR8, and TLR9, traffic between the endoplasmic reticulum and intracellular compartment such as the endosomes and the lysosomes [22]. An obvious function of these trafficking pathways is to scan for viral and/or bacterial nucleic acids, thus playing a central role in innate antiviral responses. Therefore siRNAs internalized via endocytosis are more likely to activate endosomal TLRs. It should be noted that cytoplasmic delivery of immunostimulatoty synthetic siRNAs or shRNA did not induce immune response in human blood cells [17, 21], suggesting that they are not recognized by cytolasmic sensors of ds RNAs. In this respect, a recent study has showed that endogenously expressed shRNAs are not immunostimulatory in human cells [23]. Interestingly, Williams and colleagues demonstrated that the presence of 2-base 3′-overhangs in synthetic siRNAs or in Dicer processed shRNAs blocks the activation of RIG-1, a major cytolasmic sensor for viral ds RNAs [24].

STRUCTURE FEATURES THAT ACTIVATED TLR7 AND TLR8

Initial experiments indicate that some types of secondary structures and/or specific nucleotides are responsible for the activation of NF-κB signaling pathway by siRNAs in adherent PBMC, an enriched monocyte population [10]. Monocytes are circulating peripheral blood cells that can be differentiated by cytokines into macrophages of different phenotypes as well as into dendritic cells. As mentioned above, siRNA effects are sequence dependent and can occur with ds siRNAs and ss siRNAs [20]. Thus, what is the nature of IFN-inducing motif present in one sequence but absent in another? Although GU dinucleotides were found to trigger TLR7 and TLR8 activation [25], their absolute requirement in siRNA activation of innate immunity is still not clear [21]. Judge and colleagues identified one RNA motif and its immunostimulatory effect seems to depend on the GU content [19]. However, Hornung and colleagues identified a second RNA motif that is recognized by TLR7 in the context of siRNA duplexes and the activity does not depend on GU content [18]. It is worth noting that several siRNA sequences without GU nucleotides activated the immune system [21, 26]. Thus, it is likely that in addition to GU dinucleotides other characteristics such as RNA structure, base position, and base composition of the siRNA flanking sequences may be involved.

2′-RIBOSE MODIFICATIONS OF URIDINES BLOCKS IMMUNE ACTIVATION

The identification of the immunostimulatory sequence that can activate innate immunity will allow the design of siRNAs to minimize activation or to increase the immune response for combating infections and tumor cells. Considering the simplicity of the immunostimulatory motifs and their high frequency in human RNAs, it is desirable to find strategies that evade immune recognition of siRNAs. At least two basic strategies can be used to block immune recognition of

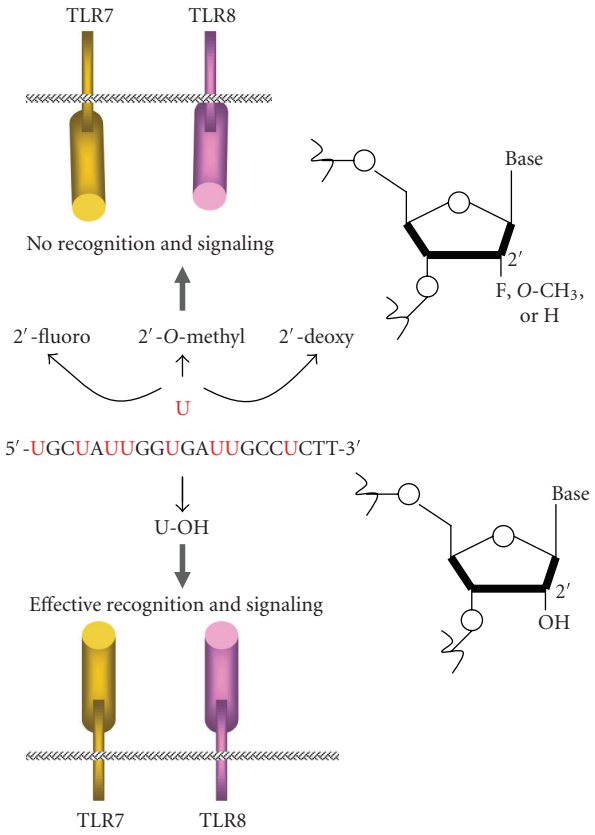

Figure 1: Unmodified siRNA duplexes and single-stranded siRNAs (sense or antisense strand) activate innate immunity through TLR7 and TLR8 resulting in cytokines and type I interferon production. 2′-ribose modifications of uridines abrogate TLR recognition of RNAs and therefore signaling [21]. Modified ss siRNAs did not compete with unmodified siRNAs to activate TLR signaling, indicating that 2′ modifications of uridines block the binding to TLRs (unpublished data).

siRNA. One is to use delivery agents that avoid the delivery and/or retention of siRNA within the endosomes. The other is to apply chemical modifications. Regarding the first strategy, Song and colleagues described an antibody-based delivery strategy that can result in gene silencing without immune activation [27]. The second strategy relies in the use of modified nucleotides, which are essential to protect siRNAs from nuclease degradation and ameliorate their pharmacokinetic parameters in vivo [13]. However, the chemical modification that blocks immune activation must be chosen carefully so as not to inhibit siRNA silencing activity. Thus, finding the appropriate chemical modifications for inhibiting siRNA immune activation will be important for exploring their therapeutic applications. In this respect, replacement of the 2′-hydroxyl uridines with either 2′-fluoro, 2′-deoxy, or 2′-O-methyl uridines abrogated immune recognition of siRNAs by TLRs [21]. Thus, endosomal TLRs can distinguish between modified and unmodified RNAs (Figure 1). In order to distinguish between self and nonself RNAs, the immune system may use both endosomal compartmentalization and RNA modification strategies [20]. Collectively, these recent findings offer the possibility of choosing the appropriate modifications that evade immune activation without reducing siRNA-silencing potency. Of note, siRNA with either 2′-fluoro uridines or 2′-deoxy uridines maintained silencing activity [26, 28].

Another potential source of toxicity is the destruction of cellular mRNAs that share partial homology to the siRNA sequences. Indeed, recent studies demonstrated that both siRNAs and microRNAs could interact with undesired target mRNAs via base pairing of only few nucleotides, leading to inhibition of gene expression [12, 29]. Interestingly, we have found that chemical modifications of siRNAs not only evade immune activation but also reduce the ss siRNAs and ds siRNA "off-target effects" [30]. Although the evading mechanism is not known, it is probable that the interaction of ss siRNAs or ds siRNAs with unintended cellular mRNAs is affected by chemical modifications.

IMMUNOSTIMULATORY siRNAs AS VACCINE ADJUVANTS

It is generally accepted that initiation of a specific immune response requires activation of innate immunity resulting in a proinflammatory response. The produced cytokines and chemokines assist in activating and directing the adaptive immune responses. Therefore, a vaccine has to induce a proinflammatory response to be effective. As discussed above, siRNA sequences containing immunostimulatory motifs induced interferons, chemokines, proinflammatory cytokines, monocyte differentiation, and dendritic cell maturation [20]. Activated DCs produce high levels of IL-12, INF-α, and proinflammatory cytokines such as IL-6 and TNF-α. IFN-α triggers not only innate immune defense such as the activation of NK cells, but also adaptive Th-1 responses, which are important for killing tumor cells and virus infected cells [20]. Engagement of endosomal TLR7 and/or TLR8 with siRNAs causes activation of at least three key transcription factors, NF-κB, IFN regulatory factor (IRF)-3, and IRF-7, which are important immune responses [30]. Previous studies have shown that the activation of TLR9 can improve both cell-mediated and humoral responses to antigens [31]. Notably, endosomal TLR9 recognize unmethylated 2′-deoxyribo (cytidine-phosphate-guanosine) CpG motifs that are more commonly found in bacterial and viral genomes [32]. Most, but not all, CpG dinucleotides are methylated in the genomic DNA of vertebrates. Although much remains to be revealed regarding the adjuvant potency of RNA oligonucleotides, the reported data indicate that ss siRNA- and ds siRNAs-containing immunostimulatory RNA motifs might improve cancer and viral vaccines.

CONCLUDING REMARKS

As with any new agent, issues of delivery and specificity are major obstacles before siRNAs can be used in patients. Similar to antisense oligonucleotides, certain synthetic siRNAs activated innate immunity via TLRs, in particular TLR7 and TLR8. Therefore, there is a need to examine

the immunostimulatory effects of any potential therapeutic siRNA in human immune cells prior to clinical applications. The recent findings clearly show that the 2′-hydroxyl uridines are absolutely essential for TLR7 and/or TLR8 recognition and signaling. Indeed, replacement of uridines with 2′-fluoro, 2′-deoxy, or 2′-O-methyl modified counterparts abrogated immune activation by ss siRNA and siRNA duplexes. Alternatively, the presence of 2′-modified uridines might protect siRNAs for being sensed by the immune system. Also, most of the other bystander effects not related to immune activation were also inhibited by chemical modifications. These findings will enable the rational design of siRNAs that avoid the induction of bystander effects.

ACKNOWLEDGMENT

We thank Dr. Anne Dybwad for critical reading of the manuscript.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 45716, Pages 1–7
DOI 10.1155/JBB/2006/45716

Review Article

Generation of RNAi Libraries for High-Throughput Screens

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Received 12 February 2006; Accepted 3 April 2006

The completion of the genome sequencing for several organisms has created a great demand for genomic tools that can systematically analyze the growing wealth of data. In contrast to the classical reverse genetics approach of creating specific knockout cell lines or animals that is time-consuming and expensive, RNA-mediated interference (RNAi) has emerged as a fast, simple, and cost-effective technique for gene knockdown in large scale. Since its discovery as a gene silencing response to double-stranded RNA (dsRNA) with homology to endogenous genes in *Caenorhabditis elegans* (*C elegans*), RNAi technology has been adapted to various high-throughput screens (HTS) for genome-wide loss-of-function (LOF) analysis. Biochemical insights into the endogenous mechanism of RNAi have led to advances in RNAi methodology including RNAi molecule synthesis, delivery, and sequence design. In this article, we will briefly review these various RNAi library designs and discuss the benefits and drawbacks of each library strategy.

INTRODUCTION

RNA-mediated interference (RNAi) provides direct causal links between specific genes and observed loss-of-function (LOF) phenotypes. RNAi is an evolutionarily conserved phenomenon in which gene expression is suppressed by the introduction of homologous double-stranded RNAs (dsRNAs). After dsRNA molecules are delivered to the cytoplasm of a cell, they are cleaved by the RNase III-like enzyme, Dicer, to 21- to 23-nt small interfering RNAs (siRNAs) [1]. These siRNA duplexes are loaded into Argonaute2 (Ago2), the catalytic component of the RNA-induced silence complex (RISC) [2]. Ago2 cleaves the passenger strand of the siRNA duplex and the antisense strand remains bound to Ago2. The antisense strand in the now mature RISC serves as a guide for sequence directed destruction of homologous mRNA, resulting in silencing of the target gene [3]. In lower organisms such as *C elegans* and *Drosophila*, RNAi is typically induced by the introduction of a long dsRNA (up to 1-2 kb) produced by *in vitro* transcription. Although the core RNAi mechanism appears to be conserved among diverse organisms, this simple approach cannot be used in mammalian cells, where introduction of long dsRNA (> 30 nt) elicits a strong antiviral response that obscures any gene-specific silencing effect [4, 5]. Much of this response is caused by activation of the dsRNA-dependent protein kinase PKR, which phosphorylates and inactivates the translation initiation factor eIF2a [6, 7]. It was not until the discovery that 21-nt siRNAs could effectively trigger the RNAi silencing response without activating the antiviral response that RNAi technology could be developed for mammalian systems [8].

Originally limited to lower organisms, RNAi technology has advanced to accommodate a variety of organisms to include mammals with methodologies that are readily adapted to high-throughput screens (HTS) [9, 10]. The present availability of commercial RNAi libraries in addition to advancements in RNAi delivery methods has provided the opportunity for genome-wide screens evaluating any biological pathway. It is crucial that when deciding on the use of RNAi technology for the purpose of a genome-wide screen that one carefully evaluates the characteristics of the selected RNAi library so that screens can be efficiently performed with excellent gene coverage and highly reproducible data. One must ensure that the RNAi library selected has been designed to maximize the efficiency of gene silencing and that the method of RNAi molecule delivery is well suited for both the type of RNAi molecule as well as the system of interest. The choice of screening an arrayed library or as pools is also another option that should be carefully considered [11]. In this article, we will review the current RNAi methodologies based on the present understanding of the RNAi biochemical process and briefly discuss developing features in library design.

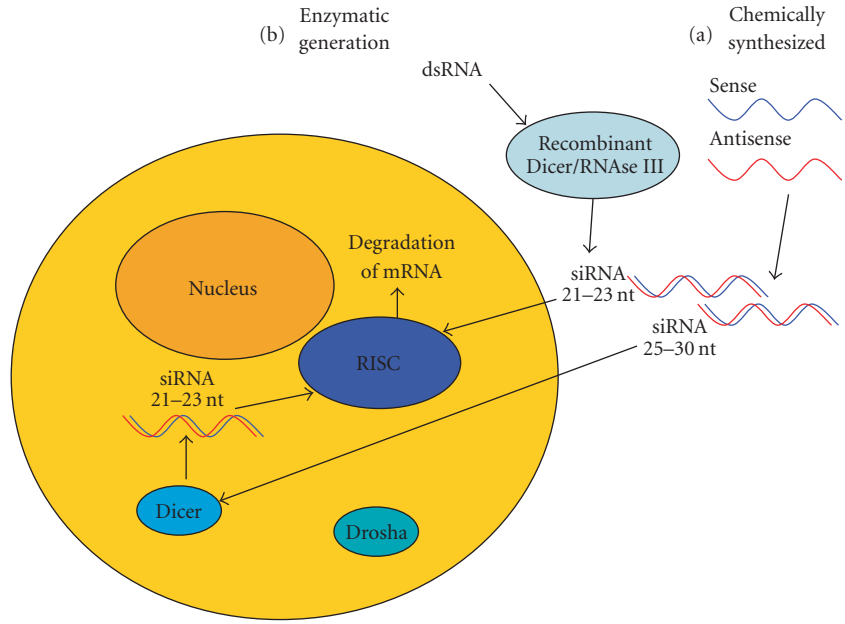

Figure 1: Extracellular generation of siRNA molecules. (a) Sense and antisense strands of RNA are chemically synthesized and annealed to form 21–23 nt or 25–30 nt dsRNA molecules. 21–23 nt siRNA molecules can directly interact with RISC and guide degradation of the corresponding mRNA. 25–30 nt dsRNA molecules must first be cleaved by Dicer to generate the 21–23 nt siRNA molecule which can be loaded into RISC. (b) Recombinant Dicer or RNAse III enzymes can also be used to generate siRNA molecules with silencing capabilities. 21–23 nt siRNA molecules are cleaved from dsRNA and associate with the RISC.

Chemical synthesis

Initial RNAi libraries were directed solely to invertebrate organism genomes and comprised of long dsRNA fragments up to 1-2 kb in size, which were generated through *in vitro* transcription. These long dsRNAs were found to be both highly specific and potent inducers of gene silencing in lower organisms, but the antiviral response in higher mammalian systems requires a different approach [8]. Since the realization that siRNAs could avoid the antiviral response while still effectively triggering a LOF phenotype, many groups began chemically synthesizing siRNAs. Chemically synthesized RNAi molecules take the form of small duplex RNA molecules. The sense and antisense strands are synthesized separately, annealed, and then delivered to cells by such means as transfection reagents, electroporation, or microinjection. Improved understanding of the RNAi mechanism has resulted in different RNAi molecule designs that enter the RNAi silencing pathway at different enzymatic points. Synthetic siRNA molecules can be designed to interact either with Dicer or RISC upon cellular entry (Figure 1(a)). Initial siRNAs were designed to resemble Dicer products 21–23 nt in size. Dicer product mimics, once transferred into the system of interest, load to RISC directly and guide the degradation of homologous mRNA immediately. Kim et al recently demonstrated that 25–30 nt in length RNA duplexes can more effectively induce gene silencing with up to 100-fold greater potency than the analogous 21-mer siRNA by first undergoing Dicer cleavage [12]. Kim et al also noted that some 27-mer duplexes were shown to effectively silence target regions refractory to the conventional 21-mer siRNA. Chemically synthesized siRNAs are more widely used in HTS for the reason of well-characterized reagents, immediate knockdown of the target mRNA, and high transfection efficiencies compared to that of plasmid-based ones.

Algorithm-based design

Initial success in knockdown with small siRNAs has since improved due to greater understanding of the silencing mechanism of Dicer and RISC. The most crucial aspect of an RNAi library directed at mammalian systems is the choice of the sequences used to target each gene due to the base pairing specificity required for precise siRNA targeting and the differential silencing potencies of individual siRNAs corresponding to distinct regions of the same mRNA [13]. Ideally, the RNAi molecule must effectively knock down gene expression while avoiding off target effects which can be either sequence-independent or sequence-specific [14]. As mentioned above, siRNAs can trigger the mammalian antiviral response inducing translation inhibition or cell death in a sequence-independent manner [6, 7, 15]. Additionally, sequence similarity to an off-target transcript can result in inadvertent degradation [14] or translation inhibition [16]. Often concentration-dependent, off-target effects can be minimized or avoided with minimal siRNA treatment and the use of unique siRNA sequences, illustrating the need for effective siRNA sequence design.

Many commercially available RNAi libraries are designed with siRNA algorithms. The design algorithms for determining siRNA sequences for mammalian genes are comprised of a number of parameters based on RNAi biochemical knowledge and empirical data for maximal silencing efficacy [17]. Some of the most common specifications [8, 18–20] include specific base compositions along the core siRNA duplex, differential base-pairing thermodynamics between the 5' sense and 5' antisense strands [20] ensuring appropriate loading of the antisense strand into RISC, A-form helix formation between siRNA and target mRNA, no internal repeats or palindromes, 30–50% GC content, and an absence of close homology to off-target gene sequences. Sequences designed by algorithms based on available genome sequence data potentially target all predicted genes and therefore would have in theory the greatest genome coverage.

The primary deficiency of the algorithm-based siRNA design is our limited understanding of the RNAi mechanism. Ideally but not practically so far, the efficacy in silencing endogenously expressed genes by algorithm-designed siRNAs in a library would be validated experimentally in cultured cells under strictly standardized conditions. In addition, the genome-wide RNAi analysis is further restricted to gene mining technology. Although gene prediction has advanced greatly and provides a good representation of the majority of genes in the genome, not all gene coding sequences [21] are identified nor are all possible splice variants predicted. Reboul et al showed that nine percent of genes identified from isolated cDNAs were not predicted by computational analysis of genome sequences [22]. The mature mRNA is the target molecule in RNAi and misprediction of gene boundaries will reduce the knockdown potential of rationally designed siRNA molecules.

To deflect such problems as misprediction and variable silencing capabilities, many libraries incorporate a degree of redundancy, using multiple designed siRNA sequences directed at a gene, to increase the likelihood of silencing the target gene. Although redundancy would have major implications in terms of various costs (eg, siRNA synthesis, screening more samples), having multiple siRNA molecules for a particular gene can be advantageous in the screen and validation phase, confirming the observed phenotype is the result of silencing of the target gene and not due to an off-target effect [23]. The availability of multiple siRNA oligos for each gene also provides the opportunity to screen as pools of oligos targeting the same gene. In certain scenarios, screening with such pools may increase the chance of knocking down the target gene expression effectively and decrease the likelihood of off-target effects (due to using lower concentration of each individual siRNA). Other concerns for synthetic siRNA libraries are its cost, stability, and nonamplifiable nature, which make the generation of siRNA libraries via chemical synthesis not financially practical in individual laboratories.

siRNAs from mRNA source

An alternative to algorithm designed synthetic oligos is the use of pools of siRNAs randomly generated with enzyme-mediated cleavage of mRNA [13, 24]. The generation of siRNA cocktails from dsRNA can be accomplished with recombinant Dicer [25] or *Escherichia coli* (*E coli*) RNase III [26] (Figure 1(b)). Dicer is the enzyme involved in cleaving long dsRNAs into 21–23 bp siRNAs in the endogenous RNAi pathway [10]. *E coli* RNase III can also be used to cleave dsRNA into effective siRNAs that are able to directly engage RISC. The use of *E coli* RNase III to generate siRNAs may be preferred due to inefficient *in vitro* cleavage by Dicer [27]. Either enzyme will process dsRNAs into a pool of siRNAs targeting multiple sites on the mRNA of interest. Calegari et al were able to knock down galactosidase expression in the developing CNS system of day 10 mouse embryos with a complex pool of siRNAs prepared from endoribonuclease digestion (esiRNA) with RNase III [24]. Yang et al were able to knock down endogenous c-myc protein levels in 293 cells by 70% with esiRNA, as well as Cdk2 expression in a dosage-dependent manner [27]. The gene silencing effect elicited by Dicer and RNase III generated pools of siRNA are comparable to well-designed individual siRNAs, but sequence rational design is not required.

RNAi expression systems

Model systems such as the *C elegans* and *Drosophila* are well adapted to chemically synthesized or mRNA-cleavage-derived siRNAs due to the presence of an endogenous amplification of the RNAi signal [1]. In lower organisms, siRNAs prime dsRNA synthesis via RNA-dependent RNA polymerase (RdRP) where the target mRNA functions as a template [28] allowing the generation of new dsRNAs. The *C elegans* model system is especially well suited for siRNA silencing not only due to endogenous amplification mechanism but also because of the phenomenon of systemic RNAi, where gene silencing can be observed in areas of the body distant from the site of the initial dsRNA delivery [29]. Systemic RNAi is due to a multispan transmembrane protein known as SID-1, which enables intercellular transport of dsRNA. This feature is not available in all lower invertebrate organisms and does not exist in *Drosophila* which has only cell-autonomous RNAi.

Mammalian systems possess neither endogenous amplification nor the phenomenon of systemic RNAi, therefore the effects of chemically synthesized RNAi molecules are limited to transient knockdown of the target gene as a consequence of cell division and/or degradation of the siRNA molecule. Most HTS experiments require only transient knockdown to sufficiently produce an observable phenotype. Transient knockdown is insufficient for groups concerned with biological processes requiring long-term gene silencing or for protocols that require some sort of selection. To address the issue of transient knockdown, many groups have elected to use intracellular expression of siRNA or short hairpin RNA (shRNA) molecules from plasmid DNA driven by either small nuclear RNA (snRNA) U6 or the human RNase P RNA H1 promoters [30] (Figure 2). U6 and H1 are RNA polymerase III (Pol III) promoters ideally suited for si/shRNA generation. Since almost all their regulation

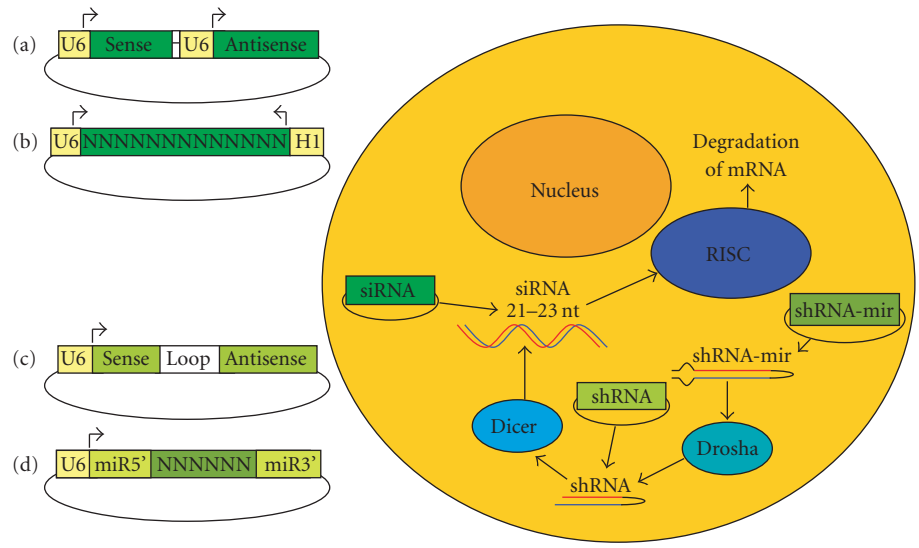

Figure 2: Intracellular siRNA molecule generation via plasmids. Dual promoter systems can direct the production of siRNA sense and antisense strands which anneal and load into RISC. (a) Tandem sense and antisense strands are driven by individual U6 promoters. (b) A single template transcribes for both the sense and antisense strand via opposing promoter design. (c) The addition of a loop structure between the sense and antisense template driven by U6 promoter generates shRNA molecules. The shRNA is cleaved by Dicer producing the functional siRNA molecule. (d) Second-generation shRNA-mir construct is based on miR-30 primary transcript driven by a single U6 promoter. The shRNA-mir molecule is first cleaved by Drosha creating an shRNA molecule recognized by Dicer thereby entering the RNAi mechanism.

elements are located upstream of the transcribed region, most insert sequences shorter than 400 nucleotides can be transcribed. The U6 promoter and the H1 promoter have the same conserved protein-binding sites and transcription termination sequence, but are different in size and identity of the +1 nucleotide, guanosine for U6, and adenosine for H1 [31].

Lee et al created an siRNA expression vector that transcribes for the sense and antisense strands (Figure 2(a)). The sense and antisense sequences were located in tandem and driven by separate U6 promoters. This tandem vector design was able to induce 90% knockdown of EGFP in 293 cells [32]. They further demonstrated their siRNA expression strategy to be capable of inhibiting HIV-1 in 293 cells showing up to 4 logs of inhibition determined via HIV-1 p24 viral antigen levels. To simplify vector construction and expression, Paul et al created a single promoter system that transcribes for the sense strand followed by a UUCG tetraloop sequence followed by the antisense strand creating an shRNA structure [33] (Figure 2(c)). The transcribed shRNA would be cleaved by endogenous Dicer and generate siRNA molecules capable of loading to RISC and guide destruction of the homologous mRNA. Verification of their vector-based shRNA expression was established with the knockdown of the human lamin A/C in HeLa cells. To further simplify library construction, a dual promoter siRNA expression vector (pDual) was developed by Zheng et al that allows the facile construction of siRNA expression library [34] (Figure 2(b)). The siRNA sequence is inserted between opposing U6 and H1 promoters and serves as the template for both the sense and antisense strand upon transfection. Zheng's construct results in an siRNA duplex with a uridine overhang on each 3' terminus, similar to the siRNA generated by Dicer which can be incorporated into the RISC without any further modification. Furthermore, a simple PCR protocol has been developed that allows an efficient and cost-effective production of siRNA expression cassettes on a genome scale in a high-throughput manner.

The vector-based shRNA design strategy was expanded by groups interested in genome-wide shRNA vector libraries. The shRNA expression construct pools can be generated from cDNA with restriction enzymes, such as DNase I [35]. Several groups have developed methods to cleave cDNA into fragments of the appropriate size and quickly clone these fragments into DNA vectors that generate shRNA structures in cells [36–38] (Figure 3). Several techniques have been reported (REGS [37], EPRIL [38], SPEED [36]) but the underlying principles guiding each are (1) restriction enzyme (RE) cleavage of cDNA into multiple fragments with nucleotide over hangs, (2) ligation of a 3' loop with *Mme*I RE recognition sequence, (3) further cleavage by *Mme*I to create fragments of the requisite size (20-21 bp), (4) conversion of dsDNA fragments into palindromic structures with PCR amplification, and (5) insertion of the randomly generated sense-loop-antisense sequences into the desired vector backbone. Shirane et al showed that their enzymatic production of RNAi library (EPRIL) generated from cDNA was able to create shRNAs which could knock down GFP and type 1 inositol 1,4,5-trisphosphate receptor 21 (IP3R) in Jurkat T cells. Pools of shRNA expression constructs, directed at both

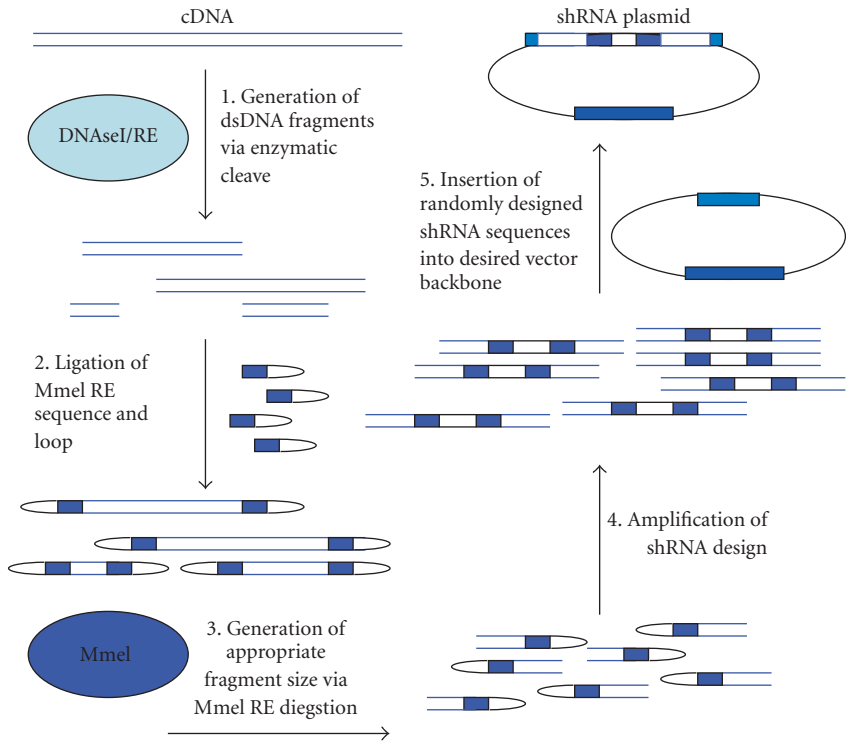

Figure 3: The generation of shRNA libraries from cDNA via enzymatic cleavage.

known and unknown genes across the transcriptome, can be generated from RE random digests of cDNA libraries [38].

More recently, advanced understanding of microRNA (miRNA) biogenesis in plants and animals has led to the construction of a second generation of shRNA expression libraries, shRNA-mir (Figure 2(d)). These shRNA-mir constructs transcribe silencing trigger molecules that mimic the natural miRNA primary transcripts. Originally believed to be transcribed from the genome as shRNAs and directly processed by Dicer [39], we now believe that miRNAs are actually transcribed into long primary polyadenylated RNAs (pri-miRNAs) [40, 41] which are first cleaved by Drosha, an enzyme in the RNase III family, to create pre-miRNAs. The pre-miRNA is then transported to the cytoplasm, mediated by Exportin-5 [42, 43], and only then recognized and cleaved by Dicer to produce a mature miRNA. Silva et al designed an shRNA-mir library, based on miR-30 primary transcript [44], which was shown to be twelve times more efficient than first-generation shRNA expression systems [45].

One added feature of using vector-based si/shRNA expression system is the facilitation of hit deconvolution by PCR amplification or barcoding when performing selective screens. Selective screens with vector libraries can be employed to fish out the target-specific and effective sequences from pools. A pooled shRNA expression library can be introduced into cells while a selective pressure is applied causing negative control cells to be eliminated from the culture [38]. The selected RNAi sequence in the resistant cells can then be determined by PCR amplification using invariant vector backbone-based primers. Alternatively, the incorporation of a gene-specific sequence into each distinct shRNA vector in the library is another means of quick identification of the selected gene target. Termed "barcode" screening [46–48], this identification sequence can be located within the vector backbone [48] or function as the short hairpin sequence of the shRNA molecule [46]. After the selection event, fluorescent dyes are attached to the barcodes which are then hybridized to microarrays, allowing for the quick identification of positive siRNA sequences within the surviving cell population.

In contrast to synthetic siRNAs, the vector-based siRNA expression systems are amplifiable and more cost-effective. However, their efficiency may be compromised in certain HTS assays. Synthetic siRNAs can directly enter the RNAi mechanism at the point of Dicer or RISC whereas vector-based RNAi molecules must first be transcribed. In addition, the transfection efficiency of plasmids may be lower relative to synthetic siRNA oligos, but for cell lines resistant to classic transfection reagents transduction with viral vectors should be considered. Furthermore, vector-based stable gene silencing may be affected by its integration position and result in a poor knockdown or off-target effects.

CONCLUSION AND FUTURE DIRECTIONS

Since the discovery of RNAi, groups have adapted this technology to suit their model system and assays of interest. A few new RNAi methodologies recently developed are advances

in viral delivery systems, incorporation of features such as inducibility, and fluorescence/selection markers. Several groups have developed adenoviral RNAi vector strategies [49, 50] in order to achieve higher levels of transduction and intracellular expression of the shRNA molecules. Lentiviral vector approaches have also been reported enabling transduction of the RNAi containing plasmids in nonproliferating cells as well as *in vivo* systems [51–53]. Inducible RNAi vectors have also been developed by several labs as both plasmid [54–56] and retro-/lentiviral vectors [57, 58]. RNAi libraries that incorporate fluorescent markers have the benefit of facilitating accurate evaluation of transfection efficiency. These library design features illustrate the adaptability of RNAi technology.

RNAi has proven to be a powerful tool in functional genomics. Its ability to induce the degradation of sequence-specific target mRNAs provides a direct relationship between a gene's expression level and its functional role [59]. RNAi-based methodologies are sufficiently robust for HTS adaptation allowing for genome-scale applications. Advancements aimed at resolving limitations as mentioned above will no doubt lead to accessibility of cost-effective, validated genome-wide siRNA collections further advancing our ability to annotate gene functions and investigate complex biological processes.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 71659, Pages 1–15
DOI 10.1155/JBB/2006/71659

Review Article

Delivery Systems for the Direct Application of siRNAs to Induce RNA Interference (RNAi) In Vivo

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Received 14 January 2006; Accepted 27 February 2006

RNA interference (RNAi) is a powerful method for specific gene silencing which may also lead to promising novel therapeutic strategies. It is mediated through small interfering RNAs (siRNAs) which sequence-specifically trigger the cleavage and subsequent degradation of their target mRNA. One critical factor is the ability to deliver intact siRNAs into target cells/organs in vivo. This review highlights the mechanism of RNAi and the guidelines for the design of optimal siRNAs. It gives an overview of studies based on the systemic or local application of naked siRNAs or the use of various nonviral siRNA delivery systems. One promising avenue is the the complexation of siRNAs with the polyethylenimine (PEI), which efficiently stabilizes siRNAs and, upon systemic administration, leads to the delivery of the intact siRNAs into different organs. The antitumorigenic effects of PEI/siRNA-mediated in vivo gene-targeting of tumor-relevant proteins like in mouse tumor xenograft models are described.

INTRODUCTION

Altered expression levels of certain genes play a pivotal role in several pathological conditions. For example, in many cancers the upregulation of certain growth factors or growth factor receptors, or the deregulation of intracellular signal transduction pathways, represents key elements in the process of malignant transformation and progression of normal cells towards tumor cells leading to uncontrolled proliferation and decreased apoptosis. Since these processes may result in the direct, autocrine stimulation of the tumor cell itself as well as the paracrine stimulation of other cells, including the stimulation of tumor-angiogenesis, many novel therapeutic strategies focus on the reversal of this effect, that is, the inhibition of these proteins or the downregulation of their expression. Likewise, several other diseases have been firmly linked to the (over-)expression of endogenous wild-type or mutated genes. Taken together, in addition to strategies based on the inhibition of target proteins, for example, by low molecular weight inhibitors or inhibitory antibodies, this opens an avenue to gene-targeting approaches aiming at decreased expression of the respective gene.

The first method to be introduced for the specific inhibition of gene expression was the use of antisense oligonucleotides in the late 1970s [1, 2]. Upon their introduction into a cell, antisense ODNs are able to hybridize to their target RNA leading to the degradation of the RNA-DNA hybrid double strands through RNAase H, to the inhibition of the translation of the target mRNA due to a steric or conformational obstacle for protein translation and/or to the inhibition of correct splicing. In the early 1980s, the discovery of ribozymes, that is, catalytically active RNAs which are able to sequence-specifically cleave a target mRNA, further expanded gene-targeting strategies [3–5]. Subsequently, both methods were extensively studied and further developed with regard to the optimization of targeting efficacies and antisense-ODN/ribozyme delivery strategies in vitro and in vivo.

Most recently, another naturally occurring biological strategy for gene silencing has been discovered and termed RNA interference (RNAi). Since RNAi represents a particularly powerful method for specific gene silencing and is able to provide the relatively easy ablation of the expression of any given target gene, it is now commonly used as a tool in biological and biomedical research. This includes the RNAi-mediated targeting in vitro and in vivo for functional studies of various genes whose expression is known to be upregulated as well as the development of novel therapeutic approaches based on gene targeting.

RNA INTERFERENCE

RNAi is an evolutionarily conserved, sequence-specific, post-transcriptional gene silencing phenomenon. It is triggered by

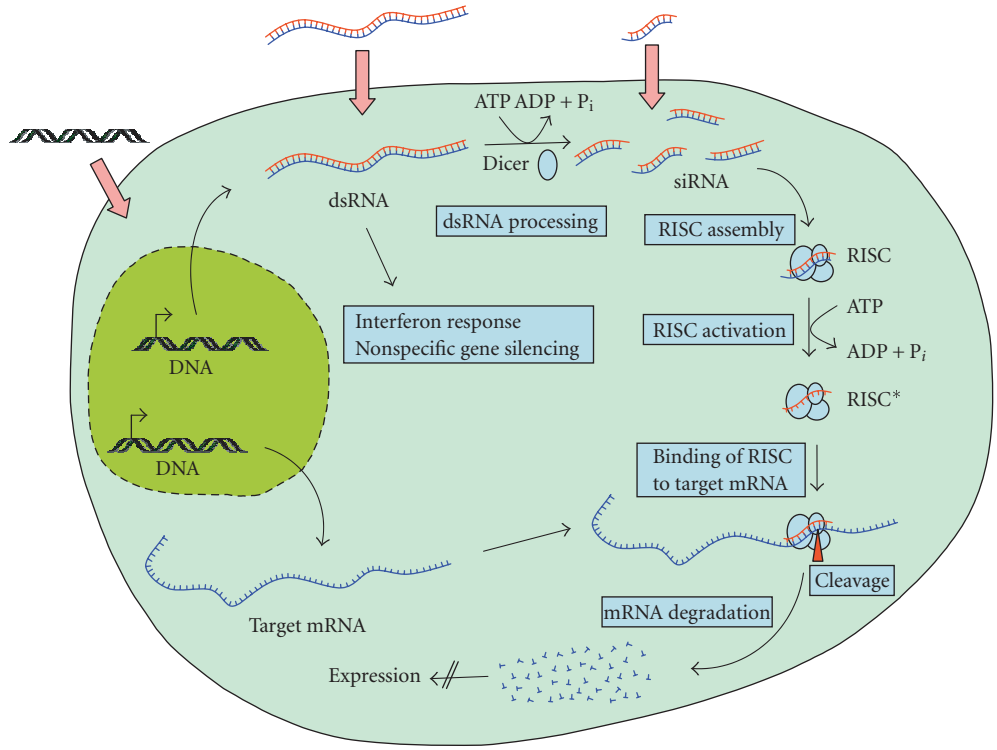

Figure 1: Mechanism of RNA interference (RNAi) in mammalian systems. Long double-stranded RNA molecules (dsRNA), which are expressed from DNA vectors (left red arrow) or directly enter the cell (center red arrow), are processed by the Dicer complex resulting in the formation of small inhibitory RNAs (siRNAs). Alternatively, to induce RNAi these small 21–23 bp duplexes are directly delivered into the cell (right red arrow). The siRNAs are incorporated into a nuclease-containing multiprotein complex called RISC, which becomes activated upon the ATP-dependent unwinding of the siRNA duplex by an RNA helicase. The now single-stranded siRNA guides the RISC complex to its complementary target mRNA which is then cleaved by the endonucleolytical activity of RISC. While the RISC complex is recovered for further cycles, the cleaved mRNA molecule is rapidly degraded due to its unprotected RNA ends.

double-stranded RNA molecules as described first in C elegans by Fire et al [6] who then introduced the name RNA interference. These findings also explained earlier observations in petunias which turned white rather than purple upon the introduction of the "purple gene" in form of dsRNA [7], and on gene silencing by antisense oligonucleotides as well as by sense oligonucleotides in C elegans [8]. Subsequent studies demonstrated that RNAi, while described under different names (posttranscriptional gene silencing (PTGS), cosuppression, quelling), is present in most eukaryotic organisms with the response to dsRNA, however, being more complicated in higher organisms.

RNAi relies on a multistep intracellular pathway which can be roughly divided into two phases, that is, the initiation phase and the effector phase. In the initiation phase, double-stranded RNA molecules from endogenous or exogenous origin present in the cell are processed through the cleavage activity of a ribonuclease III-type protein [9–12] into short 21–23 nucleotide fragments termed siRNAs. These effector siRNAs, which contain a symmetric 2 nt overhang at the 3′-end as well as a 5′-phosphate and a 3′-hydroxy group, are then in the effector phase incorporated into a nuclease-containing multiprotein complex called RISC (RNA-induced silencing complex) [13]. Several structural and biochemical studies have shed light on the processing of double-stranded RNA and the formation of the RISC complex (see, eg, [14] for a recent review). Through unwinding of the siRNA duplex by an RNA helicase activity [15], this complex becomes activated with the single-stranded siRNA guiding the RISC complex to its complementary target RNA. Upon the binding of the siRNA through hybridization to its target mRNA, the RISC complex catalyses the endonucleolytical cleavage of the mRNA strand within the target site, which, due to the generation of unprotected RNA ends, results in the rapid degradation of the mRNA molecule. With the RISC complex being recovered for further binding and cleavage cycles, the whole process translates into a net reduction of the specific mRNA levels and hence into the decreased expression of the corresponding gene. For an overview of the RNAi pathway, see Figure 1.

While from this mechanism it becomes obvious that siRNA molecules complementary to the target mRNA and thus being able to serve as a guide sequence for the RISC complex play a pivotal role in this process, they need not

be derived from long double-stranded precursor molecules. Rather, omitting the initiation phase, they can be delivered directly into the target cell (Figure 1, upper right arrow).

Several studies have led to the development of guidelines for the generation of siRNAs which are optimal in terms of efficacy and specificity [12, 16]. This includes the initial definition of the preferable length (19–25 bp) combined with a low G/C content in the range between 36% and 52% and the requirement of symmetric 2 nt overhangs at the 3′-end [16–18]. Later studies on synthetic siRNA molecules, however, revealed an up to 100-fold higher targeting efficacy in the case of even longer duplexes (25–30 nucleotides) which act as a substrate for Dicer and which therefore allow the direct incorporation of the newly produced siRNAs into the RISC complex [19]. As to be expected, intramolecular foldback structures which can result from internal repeats or palindrome sequences decrease the numbers of functional siRNA molecules with silencing capability [20]. Additional silencing-enhancing criteria include an A in position 3 and a G at position 13 of the sense strand, the absence of a C or G at position 19 and, most importantly, a U in position 10 of the sense strand. Since nucleotides 10–11 represent the site of the RISC-mediated cleavage of the target mRNA, this indicates that RISC is comparable to most other endonucleases in preferentially cleaving 3′ of U rather than any other nucleotide [20, 21]. Furthermore, it was shown more generally that the thermodynamic flexibility of the positions 15–19 of the sense strand correlates with the silencing efficacy and that the presence of at least one A/U base pair in this region improves siRNA-mediated silencing efficacy due to a decreased internal stability of its 3′-end [20].

Still, different siRNA sequences may display differing efficacies, which suggest additional still unknown criteria for optimal siRNA selection and emphasize the influence of target mRNA accessibility. In fact, several studies also correlate the siRNA efficacy with the mRNA secondary structure [18, 22–27].

In conclusion, apart from the selection criteria defined above, the individual screening of different siRNAs for highly efficient and specific duplexes, or the pooling of multiple siRNAs, is the most effective approach to increase siRNA-mediated targeting efficacy.

For the design of effective siRNAs, several algorithms on publicly accessible web sites are available (see [28] for review). To reduce the risk of nonspecific ("off-target") effects of the siRNAs, a homology search of the targeting sequence against a gene database is necessary and already incorporated in some of these web sites. Nevertheless, it has also been shown that siRNAs may cross-react with targets of limited sequence similarity when regions of partial sequence identity between the target mRNA and the siRNA exist. In fact, in some cases regions comprising of only 11–15 contiguous nucleotides of sequence identity were sufficient to induce gene silencing [29]. The prediction of these off-target activities is difficult so far.

An additional mechanism that may lead to nonspecific effects in vivo relies on the interferon system [30–33] which is induced when double-stranded RNA molecules enter a cell activating a multi-component signalling complex. This effect is particularly true for long dsRNA molecules and essentially prevents them from being used as inducers of RNA interference in mammalian systems. The development of synthetic siRNAs [10, 12, 33, 34] largely circumvents this problem since they seem to be too small. However, some synthetic siRNAs do induce components of the interferon system which seems to be dependent on their sequence [31, 32, 35] as well as, in the case of in vitro transcribed siRNAs, on the 5′ initiating triphosphate [36]. Thus, strategies to avoid as far as possible the unwanted interferon response upon application of siRNAs in vivo will include a design of siRNAs without known interferon-stimulating sequences, the use of the lowest possible siRNA dose to still achieve the desired effect and optimized siRNA delivery methods.

OLIGONUCLEOTIDE DELIVERY SYSTEMS

Based on the known mechanisms of antisense technology, ribozyme-targeting or RNAi, small oligonucleotides or plasmid-based expression vectors can be used to specifically downregulate the expression of a given gene of interest or of pathological relevance in vitro. In principle, this also applies to the in vivo situation leading to novel, potentially relevant therapeutic approaches.

For the delivery of therapeutic nucleic acids, viral vectors have been used which have the advantage of high transfection efficacy due to the inherent ability of viruses to transport genetic material into cells. On the other hand, however, viral systems show a limited loading capacity regarding that the genetic material are rather difficult to produce in a larger scale and, most importantly, pose severe safety risks due to their oncogenic potential and their inflammatory and immunogenic effects which prevent them from repeated administration [37–40].

In the light of these problems, concerns, and limitations, nonviral systems have emerged as a promising alternative for gene delivery. Main requirements are the protection of their nucleic acid "load" as well as their efficient uptake into the target cells with subsequent release of the DNA or RNA molecules and, if necessary, their transfer into the nucleus. Several strategies can be distinguished, mainly lipofection and polyfection relying on cationic lipids or polymers, respectively (see, eg, [41–43]).

The efficient protection against enzymatic or nonenzymatic degradation is particularly important for RNA molecules including siRNAs. In fact, while the therapeutic potential of siRNAs for the treatment of various diseases is in principle very promising, limitations of transfer vectors may turn out to be rate-limiting in the development of RNAi-based therapeutic strategies. One approach to solve this problem is the use of DNA expression plasmids which encode palindromic hairpin loops with the desired sequence. Upon transcription and folding of the RNA, the double-stranded short hairpin RNAs (shRNAs) are recognized by Dicer and cleaved into the desired siRNAs. Additionally, an in vitro method has been described recently which is based on the expression of shRNAs in E coli and their delivery

via bacterial invasion [44]. While all these different DNA-based systems offer the advantage of siRNA expression with a longer duration and a probably higher level of gene silencing, they still rely on (viral or nonviral) delivery of DNA molecules and again raise safety issues in vivo. Hence, the direct delivery of siRNAs molecules, derived from in vitro transcription or chemically synthesized, offers advantages over DNA-based strategies and may be preferable for in vivo therapeutic use.

In the last years, a large body of studies has been published which describe different strategies for the systemic or local application of siRNAs in vivo. Tables 1–3 give an overview. The probably largest number of papers focuses the use of unmodified siRNAs (Table 1) whose administration is often performed IV by hydrodynamic transfection (high pressure tail vein injection). While this method is widely used and in some cases led to efficient target gene inhibition in the liver and, to a lesser extent, in lung, spleen, pancreas, and kidney, it may suffer from certain technical and practical limitations at least in a therapeutical setting since it relies on the rapid IV injection of a comparably large volume (>= 1 ml/mouse/injection, in theory equivalent to a ~ 3 l IV bolus injection in man). Alternative strategies for the application of naked siRNAs include various delivery routes which, however, often provide an only local administration or rely on an administration at least close to the target tissue or target organ, thus restricting the number of target organs which may not be relevant for certain diseases. It should also be noted that several studies described here and below use rather large amounts of siRNAs and that upon intravenous injection of siRNAs the liver is the primary site of siRNA uptake. As an alternative approach for the application of siRNAs in vivo, their delivery by liposomes/cationic lipids has been described. For liposome-based siRNA formulations, a wide variety of modes of application allowing local or systemic delivery has been used (Table 2). Finally, several other strategies for local or systemic siRNA administration have been explored, including chemical modifications of siRNA molecules, electropulsation, polyamine, or other basic complexes, atelocollagen, virosomes, and certain protein preparations (Table 3).

An alternative approach relies on the complexation of unmodified siRNA molecules with a cationic polymer, polyethylenimine (PEI).

POLYETHYLENIMINES: FROM DNA TRANSFECTION TO siRNA DELIVERY IN VITRO AND IN VIVO

Polyethylenimines (PEIs) are synthetic polymers available in branched or linear forms (Figure 2, upper panels) and in a broad range of molecular weights from < 1000 Da to > 1000 kd. Commercial PEI preparations, although labelled with a defined molecular weight, consist of PEI molecules with a broad molecular weight distribution [45–47]. PEIs possess a high cationic charge density due to a protonable amino group in every third position [48, 49]. Since no quarternary amino groups are present, the cationic charges are generated by protonation of the amino groups and hence are dependent on the pH in the environment (eg, 20% at pH 7.4, see [50] for review). Due to its ability to condense and compact the DNA into complexes, which form small colloidal particles allowing efficient cellular uptake through endocytosis, PEI has been introduced as a potent DNA transfection reagent in a variety of cell lines and in animals for DNA delivery (for review, see [51, 52] and references therein). In fact, in several studies PEI has been shown to be able to deliver large DNA molecules such as 2.3 Mb yeast artificial chromosomes (YACs) [53] as well as plasmids or small oligonucleotides [48, 54–56] into mammalian cells in vitro and in vivo. The N/P ratio, which indicates the ratio of the nitrogen atoms of PEI to DNA phosphates in the complex and thus describes the amount of PEI used for complex formation independent of its molecular weight, influences the efficiency of DNA delivery. A positive net charge of the complexes, resulting from high N/P ratios, inhibits due to electrostatic repulsion their aggregation and improves their solubility in aqueous solutions as well as their interaction with the negatively charged extracellular matrix components and thus their cellular uptake [57]. Additionally, the strong buffer capacity, described by the "proton sponge hypothesis" which postulates enhanced transgene delivery by cationic polymer-DNA complexes (polyplexes) containing H^+ buffering polyamines due to enhanced endosomal Cl^- accumulation and osmotic swelling/lysis [48], seems to be responsible for the fact that PEI-based delivery does not require endosome disruptive agents for lysosomal escape. This tight condensation of the DNA molecules as well as the buffering capacity of PEI in certain cellular compartments like endosomes and lysosomes also protects DNA from degradation [48, 49, 58, 59]. PEIs have been successfully used for nonviral gene delivery in vitro and in vivo. While initial publications showed increased transfection efficacies when using high molecular weight PEIs [45], more recent studies demonstrated the advantages of certain low molecular weight PEIs [47, 60, 61]. The higher transfection efficacy of low molecular weight PEIs may be due to a more efficient uptake of the resulting PEI/DNA complexes, a better intracellular release of the DNA and/or lower in vitro cytotoxicity as compared to high molecular weight PEI [60–63]. In fact, a decrease in the molecular weight of the PEI leads to an increase in complex size which may be favourable at least for in vitro use [64, 65]. On the other hand, other PEIs with very low molecular weight (< 2 kd) display little or no transfection efficacy even at very high N/P ratios which may be attributed to the fact that a decrease in the molecular weight of PEI has been shown to translate into an increasingly lower ability to form small complexes [63]. Therefore, low molecular weight PEIs require higher N/P ratios for optimal transfection efficacies as compared to higher molecular weight PEIs since higher N/P ratios lead to an increase in compaction with reduced complex sizes and a reduced tendency of the complexes to aggregate due to hydrophobic interactions [61, 63, 64]. Nevertheless, while several parameters have been extensively studied, some precise determinants for transfection efficacy remain to be elucidated (see [50, 66] for review). Also, the mechanism of the cytotoxic

Table 1: Studies based on the direct application of siRNAs to induce RNAi in vivo: administration of unmodified siRNAs.

Administration Intravenous	Target tissue/organ	Target gene(s)	Target disease/aim of study	Reference
Hydrodynamic transfection	Liver	caspase-8	Fas-mediated apoptosis/ acute liver failure	[94]
Hydrodynamic transfection	Liver	HBsAg	Inhibition of HBV replication	[95]
Hydrodynamic transfection	Liver	HBsAg	Inhibition of HBV replication	[96]
Hydrodynamic transfection	Liver	GFP	Downregulation of GFP	[97]
Pulse injection	Liver	Fas	Fulminant hepatitis	[98]
High or low pressure	Liver	Fas	Fas downregulation in liver	[99]
Large-volume, high-speed injection	Liver	mdr1a	Downregulation of mdr1a	[100]
High-volume injection (with lipiodol)	Liver	caspase-8, caspase-3	Protection against ischemia/ reperfusion injury	[101]
Hydrodynamic transfection	Liver and limb grafts	DsRed2, GFP	Downregulation of target genes	[102]
	Metastatic breast cancer cells	CXCR4	Blockage of breast cancer metastasis	[103]
Hydrodynamic transfection	Coxsackievirus/various organs	CVB 2A	Coxsackieviral cytopathogenicity	[104]
	Pancreatic adenocarcinoma xenograft	CEACAM6	Tumor growth inhibition	[105]
	Pancreatic adenocarcinoma xenograft	EphA2	Tumor growth inhibition	[106]
	Pancreatic adenocarcinoma xenograft	FAK	Enhanced gemcitabine chemosensitivity	[107]
Hydrodynamic transfection (renal vein)	Kidney	Fas	Renal ischemia-reperfusion injury	[108]
Hydrodynamic transfection	Lung	Nucleoprotein, acidic polymerase	Influenza virus infections	[109]
Hydrodynamic transfection	Pancreas	Ins2	Downregulation of the Ins2 gene	[110]
Hydrodynamic transfection	Blood-brain barrier	Organic anion transporter 3	Brain-to-blood transport	[111]
Other delivery routes				
Intraperitoneal	Fibrosarcoma xenografts	VEGF	Tumor growth inhibition	[112]
Intraperitoneal	Subcutaneous pancreatic carcinoma xenografts	bcl-2	Growth inhibition	[113]
Local injection	Optic nerve stump	c-Jun, Bax, Apaf-1	Antiapoptosis in retinal ganglion cells	[114]
Intratracheal instillation	Lung	KC, MIP-2	Acute lung injury	[115]
Local into the liver	Liver	Luciferase	Downregulation of cotransfected luciferase	[116]
Subretinal	Eye	VEGF	Ocular neovascularization	[117]
Local injection and electroporation	Mouse joint	TNF-α	Collagen-induced arthritis	[118]
Intradermal	Antigen-presenting cells	Bak, Bax	Cancer vaccine potency	[119]
Intranasal	Nose after viral infection	RSV-P, PIV-P	Respiratory viral diseases	[120]
Intranasal	Lung	HO-1	Functional analysis in lung ischemia-reperfusion injury	[121]
Intranasal	Lung	SCV	Relief from SARS coronavirus fever	[122]
In situ perfusion/ Intravenous	Pancreatic islet	—	Detection of fluorescing siRNA	[123]
Intratumoral	Breast carcinoma xenografts	RhoA/RhoC	Inhibition of tumor growth	[124]
Intratumoral	Mammary tumor xenografts	CSF-1	Inhibition of tumor growth	[125]
Intrathecal	Brain	cation channel P2X3	Chronic neuropathic pain	[126]
Renal artery and electroporation	Kidney	TGF-β1	Glomerulonephritis	[127]
Intratracheal	Lung	Fas	Hemorrhagic shock and sepsis	[128]
Stereotactic injection to hypothalamus	Brain	Agouti-related peptide	Increased metabolic rate	[129]
Intrathecal infusion using mini-osmotic pump	Brain	Pain-related cation channel $P2X_3$	Decreased mechanical hyperanalgesia	[126]
Infusion into the ventricular system	Brain	Dopamine transporter	Temporal hyperlocomoter response	[130]
Infusion into the ventricular system	Brain	Serotonin transporter	Antidepressant-related behavioural response	[131]
Intraocular	Retinal cells/terminals in supcolliculus	APP/APLP2	Alterations of synaptic function	[132]
Intraocular	Eye	VEGFA, VEGFR1, VEGFR2	Inhibition of ocular angiogenesis	[133]
Intraocular	Eye	TGF-beta RII	Prevention of ocular inflammation and scarring	[134]

Table 2: Studies based on the direct application of siRNAs to induce RNAi in vivo: administration of siRNAs based on liposomes/cationic lipids.

Administration	Target tissue/organ	siRNA formulation	Target gene(s)	Target disease/aim of study	Reference
Intravenous	Liver metastasis	Liposomes	bcl−2	Metastasis growth inhibition	[135]
Intravenous	Kidney	Liposomes	V2R	Role of V2R in water/ sodium homeostasis	[136]
Intravenous	Subcutaneous tumor xenograft	DOPC liposomes	EphA2	Tumor growth inhibition	[137]
Intravenous	Lung	Liposomes	caveolin-1	Increase in lung vascular permeability	[138]
Intravenous/intraperitoneal	Various	Liposomes	–	Detection of FITC-labeled siRNA	[139]
Intraperitoneal	Peritoneal cavity	Liposomes	IL-12p40	Inflammation	[140]
Intraperitoneal	Peritoneal cavity	Liposomes	β-catenin	Tumor growth Inhibition	[141]
Intraperitoneal	Various	Liposomes	TNF-α	Sepsis after lipopolysaccharide injection	[142]
Transurethral	Bladder cancer	Liposomes	PLK-1	Tumor growth inhibition	[143]
Local	Ear	Liposomes	GJBR75W	Hearing loss	[144]
Subcutaneous	Subcutaneous prostate carcinoma xenograft	Liposomes	bcl-2	Tumor growth inhibition	[135]
Local (tracheal grafts)	Subcutaneous tracheal grafts	Liposomes	MIF	Decreased formation of obstructive bronchiolitis	[145]
Intracardiac	Developing vascular network of chicken embryo	Lipoplexes	GFP	Downregulation of GFP	[146]
Systemic	Prostate cancer xenografts	Cationic cardiolipin liposomes	Raf-1	Inhibition of tumor growth	[147]
Intravenous	Subcutaneous breast cancer xenografts	Cationic cardiolipin analogue	c-raf	Tumor growth inhibition	[148]
Intrathecal	Spinal cord/ dorsal root ganglia	i-Fect (cationic lipid)	Delta opioid receptor	DELT antinociception	[149]
Intratumoral	Subcutaneous HeLa xenograft	Cytofectin GSV	GFP	Downregulation of GFP	[150]
Intra-cerebroventricular	Brain	JetSI (+ DOPE)	Luciferase	Downregulation of luciferase	[71]
Intravaginal	Vagina	Oligofectamine	HSV-2 proteins	Protection from HSV-2 infection	[151]

effects of PEI complexes is only poorly understood. It may rely on the formation of large aggregates in the range of up to 2 μm which, when formed on the cell surface, impairs membrane functions finally leading to cell necrosis [60]. Clearly, there is a trend towards low molecular weight PEIs as rather nontoxic delivery reagents in vitro and in vivo, which combine high biocompatibility and reduced side-effects thus also allowing to employ larger PEI/DNA complex amounts without significant cytotoxicity.

More recently, the use of polyethylenimines has been extended towards the complexation and delivery of RNA molecules, especially small RNA molecules like 37 nt all-RNA ribozymes [67–69] and siRNAs [70] (Figure 2). While chemically unmodified RNA molecules are very instable and prone to rapid degradation, the PEI complexation has been shown to lead to an almost complete protection against enzymatic or nonenzymatic degradation. In fact, PEI-complexed siRNAs, which are [^{32}P]-labeled for better detection, remain intact in vitro for several hours even in the presence of RNase A or fetal calf serum at 37°C, while non-complexed siRNAs are rapidly degraded (Figure 3(a)). This indicates that siRNA molecules are efficiently condensed and thus fully covered and protected by PEI. Indeed, the analysis of PEI/siRNA complexes by atomic force microscopy showed the absence of free siRNAs or siRNA molecule ends and thus confirms these findings regarding an efficient complexation (Grzelinski et al, submitted). However, while the complex stability seems to be sufficient for siRNA protection with all PEIs tested (Werth et al, in press; Aigner et al, unpublished data), several of these complexes do not show any targeting efficacy at all. In fact, only when using certain polyethylenimines, PEI/siRNA complexes are efficiently delivered into target cells in vitro, where siRNAs are released and display bioactivity (Figures 1 and 2). In general and as seen before for PEI/DNA complexes (see above), the transfection efficacy is dependent on the PEI used, also indicating that the siRNA targeting efficiency mainly depends on the endocytotic uptake of the complex and/or its intracellular decomposition rather than on the in vitro complex stability. Good results were obtained with commercially available JetPEI [70] while the in vivo JetPEI from the same supplier showed only poor siRNA delivery efficacies [71]. Likewise, a novel low molecular weight PEI based on the fractionation of a commercially available polyethylenimine demonstrates high siRNA protection and delivery efficacies in vitro (Werth et al, in press). Under certain conditions, the PEI/RNA (siRNA or ribozyme)

Table 3: Studies based on the direct application of siRNAs to induce RNAi in vivo: other strategies of siRNA administration.

siRNA formulation	Target tissue/organ	Administration	Target gene(s)	Target disease/aim of study	Reference
Chemically modified	Liver and jejunum	Intravenous	apoB	Reduction of apoB and total cholesterol	[152]
Chemically modified + lipid incapsulation	Liver	Intravenous	HBV	Reduced serum HBV DNA	[153]
Electropulsation	Muscle	Intramuscular	GFP	Downregulation of GFP	[154]
Histidine-lysine complex	Breast tumor xenograft	Intratumoral	Raf-1	Breast cancer	[155]
Atelocollagen	Subcutaneous prostate carcinoma xenograft	Intratumoral	VEGF	Tumor growth inhibition	[156]
Atelocollagen	Orthotopic germ cell tumor xenograft in testes	Intratumoral	HST-1/FGF-4	Tumor growth inhibition	[157]
Atelocollagen	Bone-metastatic prostate cancer	Intravenous	EZH2	Inhibition of metastatic tumor growth	[158]
Inactivated HVJ suspension	Subcutaneous HeLa xenografts	Intratumoral	Rad51	Enhancement of cisplatin anticancer effect	[159]
Protamin-antibody fusion protein	Subcutaneous melanoma xenografts	Intravenous or Intratumoral	c-myc, MDM2, VEGF	Tumor growth inhibition	[160]
PEI complexation	Subcutaneous ovarian carcinoma xenografts	Intraperitoneal	HER-2	Tumor growth inhibition	[70]
PEI complexation	Lung	Intravenous	Influenza virus genes	Influenza virus infections	[74]
Nanoplexes (RGD-PEG-PEI)	Subcutaneous N2A neuroblastoma xenografts	Intravenous	VEGF R2	Tumor growth inhibition	[73]
TransIT-TKO (polyamine)	Nose after viral infection	Intranasal	RSV-P, PIV-P	Respiratory viral diseases	[120]
Polyamine	Myocard	Intraperitoneal	Heat shock factor 1	Abrogation of HSF-induced cardioprotection	[161]
Virosomes + cationic lipids	Peritoneal cavity	Intraperitoneal	GFP	GFP downregulation	[162]

complexes retain their physical stability and biological activity also after lyophilization ([72] and Werth et al, in press). Although the PEI transfection is only transient, data from our lab show that PEI/siRNA effects are stable for at least 7 days (Urban-Klein and Aigner, unpublished results). Finally, another study has explored the use of siRNA nanoplexes comprising of PEI that is PEGylated with an RGD peptide ligand attached at the distal end of the PEI. Again, siRNA nanoplexes protect siRNAs against serum degradation and show in vitro activity [73].

The ultimate goal is the application of siRNAs in vivo which has been explored in some studies in different mouse models. Ge et al showed that PEI-complexed siRNAs targeting conserved regions of influenza virus genes are able to prevent and treat influenza virus infection in mice. Upon IV injection, PEI promoted the delivery of siRNAs into the lungs where, either given before or after virus infection, siRNA reduced influenza virus production in the lungs [74].

Most biological effects of the systemic application of PEI-complexed siRNAs, however, have been determined in different mouse tumor models and by targeting different proteins which have been shown previously to be tumor-relevant. This includes the epidermal growth factor receptor HER-2 (c-erbB-2/neu), the growth factor pleiotrophin (PTN), and vascular endothelial growth factor (VEGF) and its receptor (VEGF R2), and the fibroblast growth factor-binding protein FGF-BP.

The in vivo administration of PEI complexed, but not of naked siRNAs, through IP or subcutaneous injection resulted in the detection of intact siRNAs even hours after injection (Figure 3(b)). Radiolabeled siRNA molecules were found in several organs including subcutaneous tumors, muscle liver, kidney and, to a smaller extent, lung and brain. It is important to note that the siRNAs were actually internalized by the tissues as indicated by the fact that blood was negative for siRNAs (Figure 3(b)).

Overexpression of the HER-2 receptor has been observed in a wide variety of human cancers and cancer cell lines. Since HER-2 displays strong cell growth-stimulating and antiapoptotic effects especially through heterodimer formation with other members of the EGFR family, its overexpression has been established as a negative prognostic factor and linked to a more aggressive malignant behaviour of tumors (eg, [75]). Consequently, HER-2 qualifies as an attractive target molecule for antitumoral treatment strategies including anti-HER-2 antibodies, low molecular weight inhibitors, or HER-2-specific gene-targeting approaches. In fact, the relevance of HER-2 (over-)expression in tumor growth has been established in several in vitro HER-2 targeting studies including the use of ribozymes [76, 78, 79] or siRNAs [80, 81].

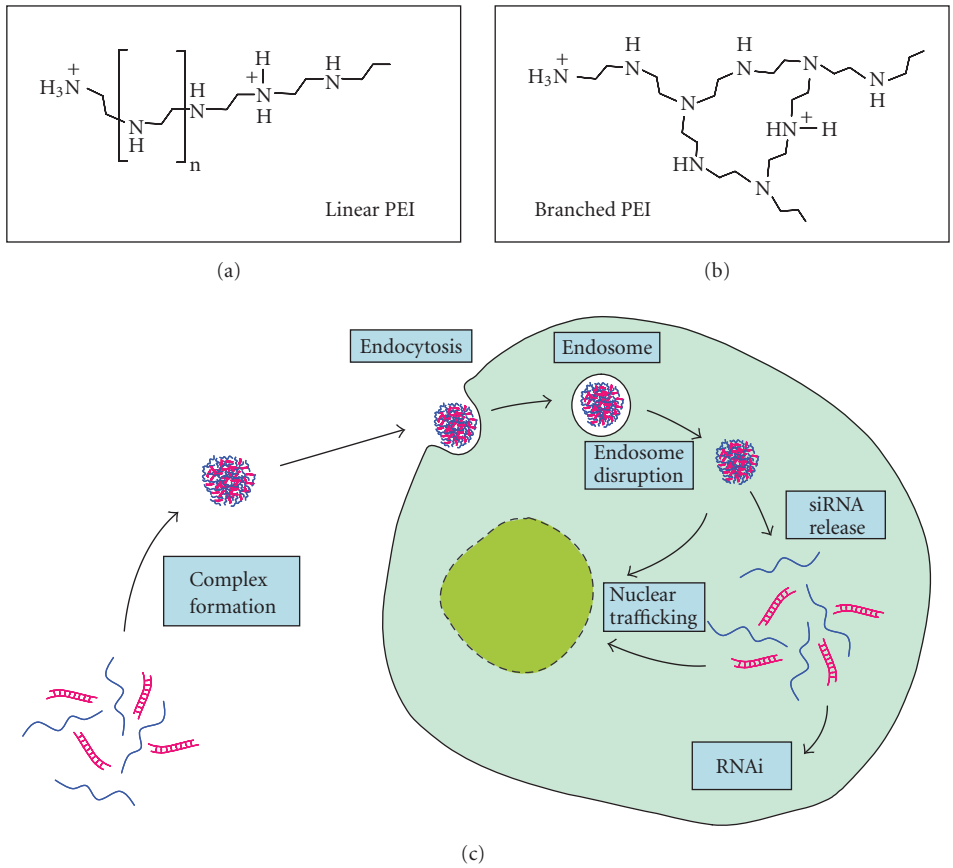

Figure 2: Polyethylenimine (PEI)-mediated siRNA transfer. Upper panel: PEIs are synthetic linear (a) or branched (b) polymers with an amino group in every third position. Dependent on the pH, some of these amino nitrogens are protonated giving PEI a high cationic charge density. Lower panel: proposed mechanism of PEI-mediated siRNA transfer. Due to electrostatic interactions, PEI is able to complex negatively charged siRNAs leading to a compaction and the formation of small colloidal particles which are endocytosed. The "proton sponge effect" exhibited by PEI complexes leads to osmotic swelling and ultimately to the disruption of the endosomes. siRNAs are protected from degradation due to their tight condensation in the complex and the buffering capacity of PEI. Upon their release from the PEI-based complex, intact siRNAs are incorporated into the RISC complex and induce RNAi (see Figure 1).

It was demonstrated that HER-2 reduction in vitro leads, among others, to the inhibition of cell proliferation and increased apoptosis.

The systemic treatment of athymic nude mice bearing subcutaneous SKOV-3 ovarian carcinoma tumor xenografts through IP injection of PEI-complexed HER-2-specific siRNAs led to marked antitumoral effects as seen by a significant reduction tumor growth (Figure 4) [70]. PEI-complexed nonspecific siRNAs or HER-2-specific, naked siRNAs had no effects. This was paralleled by the detection of intact HER-2-specific siRNAs in the tumors of the specific treatment group already 30 min after administration and for at least 4 h, and by the downregulation of HER-2 on mRNA and protein levels [70].

Another receptor, VEGF R2, was targeted in a study employing self-assembling nanoparticles based on siRNAs complexed PEI which is PEGylated with an RGD peptide ligand attached at the distal end of PEG. While the PEGylation allows steric stabilization and reduces nonspecific interactions of the complexes, the RGD motif provided tumor selectivity due to their ability to target integrins expressed on activated endothelial cells in the tumor vasculature. Upon IV administration into mice bearing subcutaneous N2A neuroblastoma tumor xenografts, a selective tumor uptake and a VEGF R2 downregulation were observed, resulting in decreased tumor growth and tumor angiogenesis [73].

The receptor ligand, VEGF, is a mitogenic and angiogenic growth factor stimulating tumor growth and angiogenesis in several tumors including prostate carcinoma. Thus, it may represent attractive target molecule for RNAi-based gene-targeting strategies also bearing in mind the double antitumoral effect due to reduction of tumor cell proliferation as well as tumor angiogenesis. The subcutaneous or intraperitoneal injection of VEGF-specific siRNAs complexed with a novel PEI obtained through fractionation of a commercially available PEI (Werth et al, in press) resulted in the reduction of tumor growth due to decreased VEGF expression levels (Höbel and Aigner, unpublished results). The same was true for PEI/siRNA-mediated targeting of FGF-BP (Dai and Aigner, unpublished results), which has been established

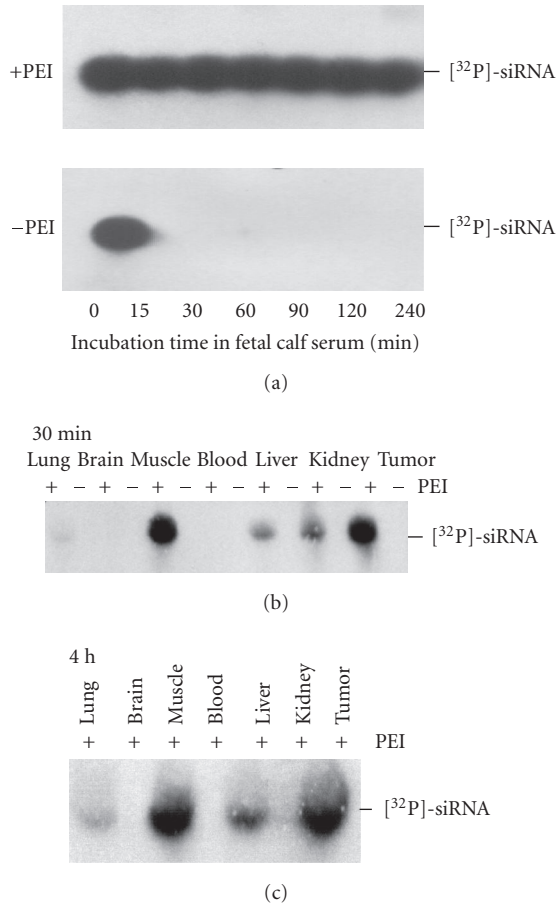

Figure 3: Protection and in vivo delivery of siRNAs upon PEI complexation. In [70] (a) in vitro protection of siRNAs against nucleolytic degradation. [^{32}P] end-labeled siRNAs, complexed (upper panel) or not complexed (lower panel) with PEI, were subjected to treatment with 1 % fetal calf serum at 37°C. At the time points indicated, the samples were analysed by agarose gel electrophoresis, blotting, and autoradiography. The bands represent full-length siRNA molecules indicating that PEI complexation leads to the efficient protection of siRNAs while noncomplexed siRNAs are rapidly degraded. (b,c) In vivo delivery of intact siRNAs upon PEI complexation. [^{32}P]-labeled siRNAs, complexed (+) or not complexed (−) with PEI, were injected IP into mice bearing subcutaneous SKOV-3 ovarian carcinoma cell tumor xenografts, and after 30 min (b) or 4 h (b) total RNA from various organ and tissue homogenates was prepared and subjected to agarose gel electrophoresis prior to blotting and autoradiography. The bands represent intact [^{32}P]-labeled siRNA molecules which for several hours are mainly found in tumor and muscle as well as in liver and, time-dependently, in kidney. Only little siRNA amounts are detected in the lung and traces in the brain.

previously as "rate-limiting" for tumor growth and angiogenesis in several tumors ([82, 83], see [84] for review).

Finally, PEI/siRNA-mediated targeting of pleiotrophin (PTN) exerted strong antitumoral effects. PTN is a secreted growth factor which shows mitogenic, chemotactic, angiogenic and transforming activity [85–93] and which is markedly upregulated in several human tumors including cancer of the breast, testis, prostate, pancreas, and lung as well as in melanomas, meningiomas, neuroblastomas, and glioblastomas. The in vivo treatment of nude mice through systemic subcutaneous or IP application of PEI-complexed PTN siRNAs led to the delivery of intact siRNAs into subcutaneous tumor xenografts and a significant inhibition of tumor growth. Likewise, in a clinically more relevant orthotopic mouse glioblastoma model with U87 cells growing intracranially, the injection of PEI-complexed PTN siRNAs into the CNS exerted antitumoral effects. This establishes, also in a complex and relevant orthotopic tumor model, the potential of PEI/siRNA-mediated PTN gene targeting as a novel therapeutic option in GBM, and further extends the modes of delivery of PEI/siRNA complexes intrathecal strategies as employed in the therapy of glioblastomas with antisense oligonucleotides.

CONCLUSION

Only a few years after their discovery, siRNAs are catching up with ribozymes and antisense oligonucleotides as efficient tools for gene targeting in vitro and, more recently, also in vivo. This includes the exploration of their potential as therapeutics which will lead to the development of siRNA-based therapeutic strategies. Their ultimate success, however, will

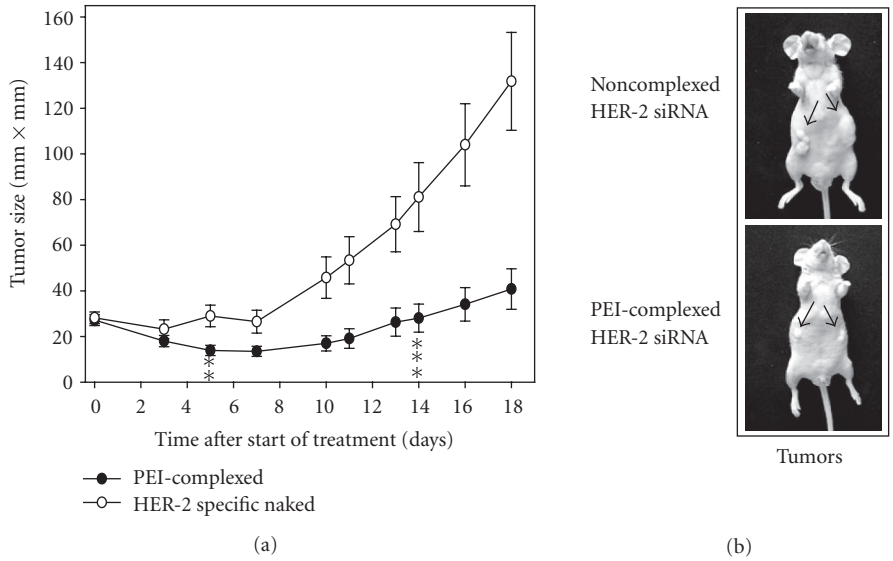

Figure 4: Systemic treatment of mice with PEI-complexed HER-2-specific siRNAs leads to reduced growth of subcutaneous SKOV-3 tumor xenografts due to decreased HER-2 expression. In [70] athymic nude mice bearing subcutaneous tumor xenografts were injected IP with 0.6 nmoles HER-2-specific naked (open circles) or PEI-complexed (closed circles) siRNAs 2–3 times per week and tumor sizes were evaluated daily from the product of the perpendicular diameters of the tumors. Mean +/- standard error of the mean (SEM) is depicted and Student's unpaired t test was used for comparisons between data sets ($^{**}P < .03$, $^{***}P < .01$). Differences in tumor growth reach significance at day 5 indicating the antitumoral effects of the PEI-complexed HER-2-specific siRNAs.

strongly depend on the development of powerful and feasible siRNA delivery strategies which need to address several issues including the stability/stabilization of siRNA molecules while preserving their efficacy and maintaining their gene-silencing activity, an efficient delivery into the target organ(s) as well as a sufficiently long siRNA half life in the organism and particularly in the target organ. Thus, siRNA delivery strategies must provide siRNA protection and transfection efficacy, the absence of toxic and nonspecific effects, they must be efficacious also when using small amounts of siRNAs and must be applicable in various treatment regimens and in various diseases even when this requires to overcome biological barriers after their administration to reach their target tissue or target organ. The research done on DNA-based gene delivery, ribozyme-targeting, and antisense technology will facilitate this process since it already provides a basis of established technologies. This is also true for the complexation of siRNAs with polyethylenimine, which may represent a promising avenue for siRNA applications in vivo. This may eventually lead to novel therapeutic strategies.

ABBREVIATIONS

dsRNA, double-stranded RNA,
FGF-BP, fibroblast growth factor-binding protein,
GFP, green fluorescent protein,
HER-2, human epidermal growth factor receptor-2,
IP, intraperitoneal,
ODN, oligodeoxynucleotide,
PEI, polyethylenimine,
PTN, pleiotrophin,
RISC, RNA-induced silencing complex,
RNAi, RNA interference,
siRNA, small interfering RNA,
shRNA, short hairpin RNA.

ACKNOWLEDGMENTS

The work of A. Aigner is supported by the Deutsche Forschungsgemeinschaft (AI 24/5-1) and by the Deutsche Krebshilfe. The author would like to apologize to the authors whose primary works have not been cited due to length considerations.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 63675, Pages 1–9
DOI 10.1155/JBB/2006/63675

Review Article

Targeted Delivery of siRNA

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Received 16 February 2006; Revised 2 May 2006; Accepted 2 May 2006

Therapeutic application of siRNA requires delivery to the correct intracellular location, to interact with the RNAi machinery within the target cell, within the target tissue responsible for the pathology. Each of these levels of targeting poses a significant barrier. To overcome these barriers several strategies have been developed, such as chemical modifications of siRNA, viral nucleic acid delivery systems, and nonviral nucleic acid delivery systems. Here, we discuss progress that has been made to improve targeted delivery of siRNA in vivo for each of these strategies.

INTRODUCTION

The functional mediators of RNA interference (RNAi) are small interfering RNAs (siRNA) [1, 2]. These double-stranded RNA molecules are typically 19 to 23 nucleotides in length, and consequently have a molecular weight of approximately 13 to 15 kd and 38 to 46 negative charges. As a consequence, passive transport over the lipophilic cell membrane is poor [3–5]. At the same time, intracellular entry and translocation into the cytoplasm (and/or nucleus), where the RNAi machinery is located, is a prerequisite, for gene silencing activity [6–9]. More importantly, for in vivo applications, intracellular entry into the target cell within the diseased tissue is required and should lead to appearance in the cytoplasm to silence the mRNA of interest (Figure 1). Ideally, siRNA should therefore be targeted to three levels: to the target tissue, the target cell type, and the subcellular compartment.

Primary obstacles for achieving this in vivo include competitive uptake by nontarget cells, excretion in urine, degradation by nucleases, and endosomal trapping.

Some literature reports claim entry of siRNA in the target cells of the target tissue after intravenous injection [10]. The observations have been attributed to translocation of siRNA over the cell membrane by a dsRNA-receptor, referred to in *Caenorhabditis elegans* as SID-1,which is responsible in this organism for systemic spreading of the silencing effects [11]. Indeed, overexpression of the mammalian homologue increases the intracellular uptake of siRNA [12]. In contrast, "naked" siRNA is used by many researchers as a negative control which fails to produce silencing effects after injection in vivo and even after prolonged incubation of cells with high siRNA concentrations in vitro. This lack of activity of "naked" siRNA indicates that not all cell types express (enough of) the SID-1 homologue, to observe silencing effects. In addition, the rapid removal of "naked" siRNA after intravenous administration from the circulation, with more than 99% of the injected dose renally excreted and taken up by liver Kupffer cells within minutes, makes a very small percentage of the administered dose available for the target tissue. This small percentage is additionally subject to nuclease degradation. Therefore, intravenous injection of naked siRNA relying on passive targeting of the diseased tissue, and SID-1 homologue-mediated target cell uptake seem to be inefficient and as yet unpredictable.

Local injection at the site of pathology avoids many of the difficulties encountered after intravenous administration, most notably the rapid elimination, and is therefore a popular approach to increase target tissue concentrations of siRNA. With this approach chances of obtaining sufficient intracellular levels of siRNA for therapeutic effects are increased [13, 14]. Furthermore, helper molecules (like cationic lipids or polymers) or physical methods (like electroporation, sonoporation, or hydrodynamic pressure) can be employed to facilitate intracellular entrance of siRNA [13, 15–19]. In addition, local production of siRNA by genes encoding for short hairpin RNA (shRNA) can ensure prolonged levels of the dsRNA intracellularly [20, 21]. The encoding genes can be delivered by viral vectors or one of the aforementioned nonviral methods.

Unfortunately, local administration is not always feasible because the target tissue cannot be reached, or covers an area that is too large to be feasible for a local injection protocol. In addition, using local injection (possibly supplemented with helper molecules or physical stimuli) selectivity in delivery to nontarget and target cell types has usually not been taken into account. This is an important aspect when considering the nonspecific effects that can be induced by dsRNA. Over the past few years, it has become clear that cells can respond strongly to siRNA by different proinflammatory reactions depending on cell type, siRNA sequence, and intracellular location [22–24]. These effects can be intensified by employing cationic helper molecules [25], an effect probably mediated by a change in the intracellular trafficking of the dsRNA [23]. As a consequence, nontarget cells may also take up siRNA and respond with induction of proinflammatory pathways in addition to the therapeutic RNAi effects within the target cells at the diseased site.

In this review we will focus on strategies for targeted siRNA delivery that are designed to improve accumulation of siRNA at three in vivo levels of delivery: at the target tissue, the target cell, and at the intracellular target site of action. We will concentrate on delivery approaches for systemic administration as such systems have broadest applicability. Three approaches will be discussed:

(1) chemical modifications of siRNA,
(2) viral nucleic acid delivery systems,
(3) nonviral nucleic acid delivery systems.

Most attention will be given to in vivo delivery strategies, as in vitro targeting studies often do not represent a fair evaluation of the many barriers that exist in vivo, possibly impeding efficient and site-specific delivery (eg, phagocyte uptake, uptake by competing cell types, excretion, intracellular processing, and siRNA (delivery system) stability).

CHEMICAL MODIFICATIONS

The nuclease sensitivity and poor tissue distribution/membrane permeation qualities of siRNA provide a reason to investigate possible chemical modifications that would improve these characteristics which would not interfere with the silencing efficiency of the molecules. Several strategies have been proposed to improve nuclease resistance and target cell uptake.

Increasing nuclease resistance

Chemical modifications in the nucleobases, sugars, and the phosphate ester backbone of siRNA can reduce siRNA sensitivity to nucleases [26–28]. Initial studies centered on the tolerance of the RNAi system for modifications in the two RNA strands [29]. A number of chemical modifications have been proposed to increase nuclease resistance, like boranophosphates [30], 4′-thioribonucleosides, phosphorothioates, 2′deoxy-2′ fluorouridine, 2′-O-methyl, 2′-O-(2-methoxyethyl), and locked nucleotides [31–36]. All of these chemically modified siRNAs were still able to induce siRNA-mediating gene silencing provided that the modifications were absent in specific regions of the siRNA and included to a limited extent. These specific restrictions regarding position and degree of modifications were dependent on the characteristics of the incorporated modification. Although increased nuclease resistance of siRNA would be expected to increase in vivo silencing effects, Layzer et al showed that this is not necessarily the case. They studied silencing effects in the liver after hydrodynamic injection, and showed that unmodified siRNA had a similar potency as the stabilized version [36].

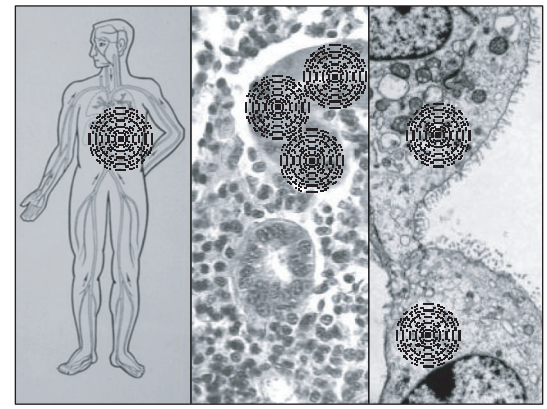

Figure 1: Three levels of targeting: preferably, siRNA should be targeted to the diseased tissue (I). Within this tissue it should be delivered to the correct cell type for silencing the mRNA of interest (II). Following entry of the target cell, siRNA should be delivered to the cytoplasm (and/or nucleus) to interact with the components of the RNAi machinery (III).

In contrast, chemical modifications were shown to enhance therapeutic effects in a mouse model of hepatitis B virus infection. Chemically modified siRNA designed against a conserved region of the hepatitis B virus was shown to decrease viral DNA, hepatitis B surface antigen levels in serum, as well as viral RNA levels in liver over 1000-fold as compared to chemically modified control siRNA and buffer-treated animals in a hydrodynamic injection protocol. The benefit of chemically modified siRNA was supported by the fact that indicators of viral infection were 30-fold higher in animals treated with unmodified siRNA [37].

Increasing intracellular uptake

Several approaches have been developed where chemical modifications have been introduced to enhance intracellular uptake of siRNA. Liao and Wang developed poly-2′-O-(2,4-dinitrophenyl) modified siRNA. The enhanced lipophilicity of this siRNA allows passive diffusion over the cell membrane, while at the same time enhancing nuclease resistance. This approach has only been investigated in vitro and shows that chemical modification can enhance siRNA potency at both fronts. As a result the silencing effects of the chemically modified siRNA specific for insulin-like growth factor receptor were strongly enhanced as compared to unmodified siRNA [38].

In a similar setup, membrane permeant peptides (penetratin and transportin) were coupled to siRNA to facilitate their intracellular uptake. By coupling the peptide via a reducible disulfide linker, the bulky peptides are expected to be cleaved-off liberating the siRNA in the cytoplasm. The approach was validated in vitro, thus supporting wide application of the basic technology. Nevertheless, cell specificity is lacking [39].

Probably the furthest developed work on chemically modified siRNAs has been reported by Soutcheck et al [40]. They have developed siRNAs with partial phosphorothioate backbone modifications and 2′-O-methyl sugar variations on the sense and antisense strands to promote nuclease resistance, while at the same time cholesterol was conjugated to the 3′ end of the sense strand using a pyrrolidine linker to change tissue distribution. The cholesterol-modified siRNA silenced reporter gene expression in vitro in the absence of transfection agents, something not observed for unmodified siRNAs. Probably the interaction of the cholesterol with serum components in the culture medium improved siRNA translocation over the cellular membrane. The cholesterol modification particularly enhanced binding of siRNA to serum albumin, probably explaining the prolonged circulation half-life measured after intravenous injection as compared to unmodified siRNA. In addition to a prolonged presence in the circulation, cholesterol-modified siRNAs were detected in liver and jejunum at concentrations of 100–200 ng per gram tissue 24 hours after the last injection of 50 mg/kg doses. These levels were sufficient to reduce the levels of the apolipoprotein apoB-100 in plasma by 31–68%. This reduction was paralleled by a 37% reduction in overall cholesterol levels, and reduction in high-density lipoprotein, low- density lipoprotein, and chylomicron levels. Despite these impressive results using relatively simple modifications, the doses of chemically modified siRNAs needed are relatively high and seem to underline that changing tissue distribution of siRNA in favor of target cell uptake by conjugation with comparatively small chemical groups is difficult to achieve. At the same time, small molecular weight modifications seem to be needed to preserve correct interaction with the RNAi enzymes. Cleavable linkers for coupling of bulkier modifications may be an approach to avoid these problems. In conclusion, chemical modifications do promise important advances regarding nuclease resistance and reduced induction of the stress response. Invitrogen has developed a second-generation siRNA, known as Stealth RNAi, in which chemical modifications are designed to increase the specificity of RNAi effects by allowing only the antisense strand to efficiently enter the RNAi pathway and eliminating induction of interferon-related pathways. Others have also demonstrated that sequence and modifications can strongly influence intensity of silencing efficiency and inflammatory reactions, providing tools to optimize these [41, 42].

Taken together, chemical modifications can markedly increase nuclease resistance of siRNA improving cellular persistence and conjugation with translocating/hydrophobic functional groups can increase membrane permeation. Strategies to affect tissue distribution profiles of siRNA with chemical modifications seem more difficult.

VIRAL NUCLEIC ACID DELIVERY

Viruses are at present the most efficient gene delivery vectors. After cell binding they are capable of delivering their nucleic acid payload intracellularly in a proficient way along with nuclear localization. Although virus-mediated delivery methods are usually based on delivery of genes encoding shRNA, few approaches used viruses to deliver chemically synthesized siRNA in vivo [43, 44].

Delivery of chemically synthesized siRNA

In this approach, reconstituted viral envelopes derived from influenza virus are used to encapsulate and deliver siRNAs. The reconstituted membrane vesicles contain the influenza virus spike protein hemagglutinin and additionally added cationic lipids. This protein is responsible for binding to and fusion with cellular membranes. The siRNA-loaded vesicles are taken up by receptor-mediated endocytosis, and are able to escape endosomal degradation by fusion with the endosomal membrane. Functional siRNA delivery was demonstrated in vitro, while in vivo uptake by macrophages in the peritoneal cavity was demonstrated after intraperitoneal injection. A similar approach, described siRNA delivery by simian virus SV40-based particles in vitro in lymphoblastoid cells [44]. As with many viral approaches, drawbacks of the systems are the difficulties of repeated administration and limited control over transduced cell type.

Delivery of DNA encoding siRNA/shRNA

A number of studies investigated the use of DNA encoding for shRNA delivered by viruses for gene silencing in vivo. Intravenous injection of 5×10^9 plaque forming units (pfus) recombinant adenovirus expressing shRNA targeting hepatitis B virus transcripts in mice with active replication of the hepatitis B virus, showed almost complete inhibition of viral protein production [45]. This in turn led to arrest of viral replication at day 17 after viral infection. The inhibitory effect persisted for at least 10 days. Interestingly, there appeared to be a fraction of viral protein that was not susceptible to RNAi-mediated silencing, which is suggested to be attributable to protection through binding of their mRNA to specific proteins. The exact nature of this protection and its possible involvement in RNAi resistance remains to be determined.

Uchida et al used expression of two separate siRNA strands against survivin by adenoviral transduction to inhibit tumor growth. Survivin is a protein that inhibits cancer cell apoptosis. Mice bearing subcutaneous U251 glioma tumors were treated with intratumoral injections of 10^{10} viral particles on three consecutive days every twenty days, ultimately leading to four-fold smaller tumors at day 48 after start of treatment as compared to empty adenoviral vector and adenoviral vector expressing irrelevant siRNA [20].

These studies demonstrate the possibilities for single intravenous or multiple local injections of virally delivered DNA encoding si/shRNA for gene silencing. This strategy has been further confirmed in a number of different in vivo models and with a number of different vectors, like intracranial delivery of lentivirus-produced shRNA for inhibition of reporter gene expression in cortical neurons [46], intraperitoneal delivery of lentivirus-produced shRNA for inhibition of viral cyclin to prevent primary effusion lymphoma in mice [47], intramuscular or intraspinal delivery of lentivirus-produced shRNA for inhibition of mutant SOD1 in amyotrophic lateral sclerosis [48, 49], and ex vivo delivery of lentivirus-produced shRNA for inhibition of CC-chemokine receptor 2 in hematopoietic cells in mice [50].

Taken together, the viral DNA-based sh/siRNA delivery process is very efficient: binding to the target cell surface and subsequent transduction, carrier stability, and protection against nucleases appear satisfactory [51–54]. However, as the discussed approaches illustrate, viruses usually lack selectivity for the target cell type. To improve specificity, the natural tropism of viruses for certain cell types may be used. Currently, much attention is focused on redirecting the natural preferred cell type of viruses towards therapeutically interesting receptors on the surface of target cells. Examples include the retargeting of murine coronavirus to the human epidermal growth factor receptor [55], directing adenovirus via fibroblast growth factor ligand towards its associated receptor (FGFR1) for delivery to glioma, or adenoviral delivery to angiogenic endothelium via RGD-peptides binding alpha v-integrins [56]. However, such approaches have not been tried as yet in combination with RNAi-mediated gene silencing in vivo.

The strength of the viral delivery approach is the efficient transduction of cells. Challenges that remain are the control over transduced cell type, especially after systemic administration. In addition, inflammatory reactions, immunogenicity, and oncogenic transformations continue to be important safety considerations for viral vectors that need to be addressed [57, 58].

Nonviral nucleic acid delivery

Whereas viral vectors possess many of the desired characteristics for efficient nucleic acid delivery, nonviral vectors possess several advantages. Important benefits of synthetic vector systems are the safety (related to their lack of immunogenicity and low frequency of integration) and ease of large-scale production. In addition, they can accommodate a wide variety of nucleic acid sizes and they allow easy modification. On the downside, transfection efficiency can be a limiting factor.

To face this weakness, many functional groups need to be incorporated into nonviral nucleic acid delivery systems. A cationic functional group is usually required to bind and condense the nucleic acid, thereby protecting it against nucleases and (important for siRNA) increasing the apparent molecular weight above the renal clearance cut-off. In addition, some cationic compounds are being used as endosomal escape enhancers. Due to the resulting positive charge, complexes tend to form aggregates by binding in the blood stream to negatively charged biomolecules. As a result, their clearance is usually rapid. Moreover, such cationic complexes possess a propensity to interact with virtually any cell type they encounter, creating a need to insulate the interactive surface of the particle to promote specificity. For that purpose, shielding groups can be added to enhance colloidal stability and reduce surface charge thereby avoiding nonspecific cell uptake. To restore cell interaction in a target-specific manner targeting ligands can be coupled to induce site-specific binding and uptake. In the case of delivery of DNA encoding for shRNA by non-viral delivery systems, nuclear translocation of the DNA is often inadequate. As such, the cytoplasmic site of activity of chemically synthesized siRNA provides an important advantage.

Delivery system based on RNA

A system consisting completely of RNA was proposed by Guo et al [59]. Their system is based on the packaging RNA of the DNA-packaging motor of bacteriophage phi29, which can spontaneously form dimers via interlocking right- and left-hand loops. By attaching the siRNA to one loop and an RNA aptamer to CD4 to the other, a cancer cell targeted system was created that could silence survivin gene expression in vitro. Alternatively, the system could also be targeted by folate.

Cationic delivery systems

Unshielded, untargeted complexes of siRNA with cationic polymers or lipids, can provide local or systemic transfection of a sufficient number of target cells for therapeutic effects. Several studies employed cationic lipids to complex siRNA to silence, amongst others,TNF-alpha in intraperitoneal macrophages after intraperitoneal administration [60], delta opioid receptor in spinal cord and dorsal root ganglia after intrathecal administration [61], polo-like kinase-1 in bladder cancer after intravesical administration, and c-raf-1 in prostate cancer cells after intravenous administration [62]. Although, a sufficient number of cells must have been reached as silencing is observed, it is fair to assume that a large part of the dose will arrive in nontarget cells. In view of the nonspecific effects that can be induced by cationic lipids themselves and in particular in combination with dsRNA, this may severely hamper therapeutic application [25, 63].

A variety of other cationic compounds have also been investigated for siRNA-delivery purposes. A linear low molecular weight form of the cationic polymer poly(ethylene imine) (PEI) has been used for treatment of (subcutaneously implanted) ovarian carcinoma in mice [64]. After intraperitoneal administration complexed siRNA was primarily recovered from muscle, liver, kidney, and tumor. Interestingly, the major organ where PEI nucleic acid-complexes are usually recovered, the lung, was largely avoided. Importantly, silencing of Her-2 with these polyplexes inhibited ovarian carcinoma growth in vivo.

Atelocollagen (a highly purified type-I collagen of calf dermis digested by pepsin), was shown to be a suitable vehicle for local delivery of siRNA [17, 65]. In addition, when administered intravenously, atelocollagen-siRNA was able to localize at sites of tumor mestastases and inhibit metastasis outgrowth [66]. More specifically, tumor levels increased ~ 6-fold as compared to levels after "naked" siRNA administration (from 0.7 to 4.3 ng/mg after injection of 25 μg siRNA). This effect was, albeit less pronounced, also seen in the other organs investigated (ie, liver, lungs, kidneys, and spleen) demonstrating that the enhanced tissue uptake is not exclusively tumor-specific. Nevertheless, delivery of these levels of siRNA silencing EZH2 (enhancer of zest homologue-2, a gene overexpressed in hormone-refractory metastatic prostate cancer) or p110-α (a phosphatidylinositol 3-kinase regulating cell survival, proliferation, and migration) resulted in strong inhibition of growth of bone metastases of prostate cancer cells. Importantly, siRNA-atelocollagen complexes failed to induce nonspecific proinflammatory responses like secretion of IFN-α and IL-12.

Targeted cationic delivery systems

A targeted amino-acid-based system was based on the cationic peptide protamine [67]. To the system's protamine-block the C-terminus of the heavy chain Fab fragment of an HIV-1 envelope antibody was coupled to form a protein construct known as F105-P. This system was highly efficient in binding to and transfection of cells expressing HIV-envelope protein, although it is unclear why the HIV-envelope protein would be internalized. Importantly, expression of interferon-β, 2′, 5′-oligoadenylate synthetase, and Stat-1, as indicators of nonspecific effects, were not increased upon siRNA transfection of HIV-envelope-expressing melanoma cells. In addition, when these cells formed subcutaneous tumors in vivo, 30% of cells took up fluorescent siRNA when delivered by F105-P after intravenous administration. Naked siRNA was not taken up, nor was F105-P-siRNA delivered to cells that were envelope-protein negative. Delivery of a combination of siRNAs against c-myc, MDM2, VEGF strongly inhibited tumor growth in vivo when delivered using the F105-P system. This combination of siRNAs attacking the tumor at multiple fronts is an important advantage of the siRNA technology as it allows simultaneous interference with a number of different pathways, while the delivery problem for each individual drug molecule (siRNA) remains the same. The versatility of this targeted system was demonstrated by exchanging the HIV-envelope antibody for an ErbB2-antibody changing the specificity of the system to ErbB2-positive breast carcinoma cells.

In a cationic lipid-based approach, Pirollo et al coupled a transferrin receptor single chain Fv region antibody fragment to the surface of cationic DOTAP: DOPE complexes containing siRNA [68]. They evaluated the targeting potential of these systems in different murine tumor models: an orthothopically implanted pancreatic carcinoma (that produced spontaneous metastases), an orthotopically implanted prostate carcinoma, and intravenously administered breast carcinoma cells giving rise to metastases in the lung. In all these models, specific accumulation of fluorescently labeled siRNA complexed to the targeted cationic lipid particles at the site of the malignancy could be demonstrated as compared to surrounding normal tissue and liver. The question whether targeted delivery resulted in gene silencing was not addressed.

Shielded targeted cationic delivery systems

Targeted cationic systems have the important advantage that they possess a recognition signal for specific interaction with the target cell type. However, the cationic surface may also be able to interact with biomolecules or nontarget cells. As such, shielding of the cationic surface may further enhance target cell specificity by reducing nontarget tissue uptake and may additionally increase colloidal stability of the siRNA complexes.

In our studies we focused on the cationic polymer PEI coupled to PEG as shielding polymer. To the distal end of the PEG-chain a cyclic RGD-peptide was coupled. This peptide is a high-affinity ligand for alpha v-integrins that are overexpressed on angiogenic endothelial surfaces [69]. Tissue distribution studies in vivo of fluorescently labeled siRNA in subcutaneous neuroblastoma-bearing mice showed that injection of "naked" siRNA did not produce appreciable tumor levels, but rather rapid clearance into the urine. PEI-siRNA complexes also lacked the production of high fluorescence in the tumor, but did increase liver and especially lung levels. The fluorescence appeared punctuate in both latter tissues, probably reflecting formation of aggregates in the circulation.

When the PEG-shielded, targeted nanoparticles were used, a higher level of specificity for the tumor and lower levels of fluorescence in the lung and liver were observed. In a therapeutic setting, siRNA against murine VEGF receptor-2 was used, since the receptor is one of the driving factors of tumor angiogenesis. Delivery to host tumor endothelium is required to inhibit tumor proliferation. Efficacy studies with VEGFR2-specific siRNA complexed in RGD-PEG-PEI nanoparticles resulted in strong inhibition of sc neuroblastoma growth rate, which was sequence-specific. These experiments suggest that the targeted shielded nanoparticles indeed deliver the siRNA to the angiogenic endothelial cells. In line with these findings, the reduced tumor growth rate was paralleled by a reduction in blood vessels in the periphery of the tumor and changes in vascular morphology of remaining vessels, supporting an antiangiogenic mechanism of action. These results were supported by studies in a model of pathological angiogenesis in the eye [70], again demonstrating vasculature-specific delivery and inhibition of angiogenesis leading to therapeutic effects. Importantly, the studies in the eye also showed that combining siRNAs against different driving factors in the VEGF-pathway in the same delivery system improved therapeutic effects. Attacking the various receptors and growth factors simultaneously seems to offer advantages. Especially in multifactorial diseases, where functional redundancy is likely, this cocktail approach seems to offer important benefits.

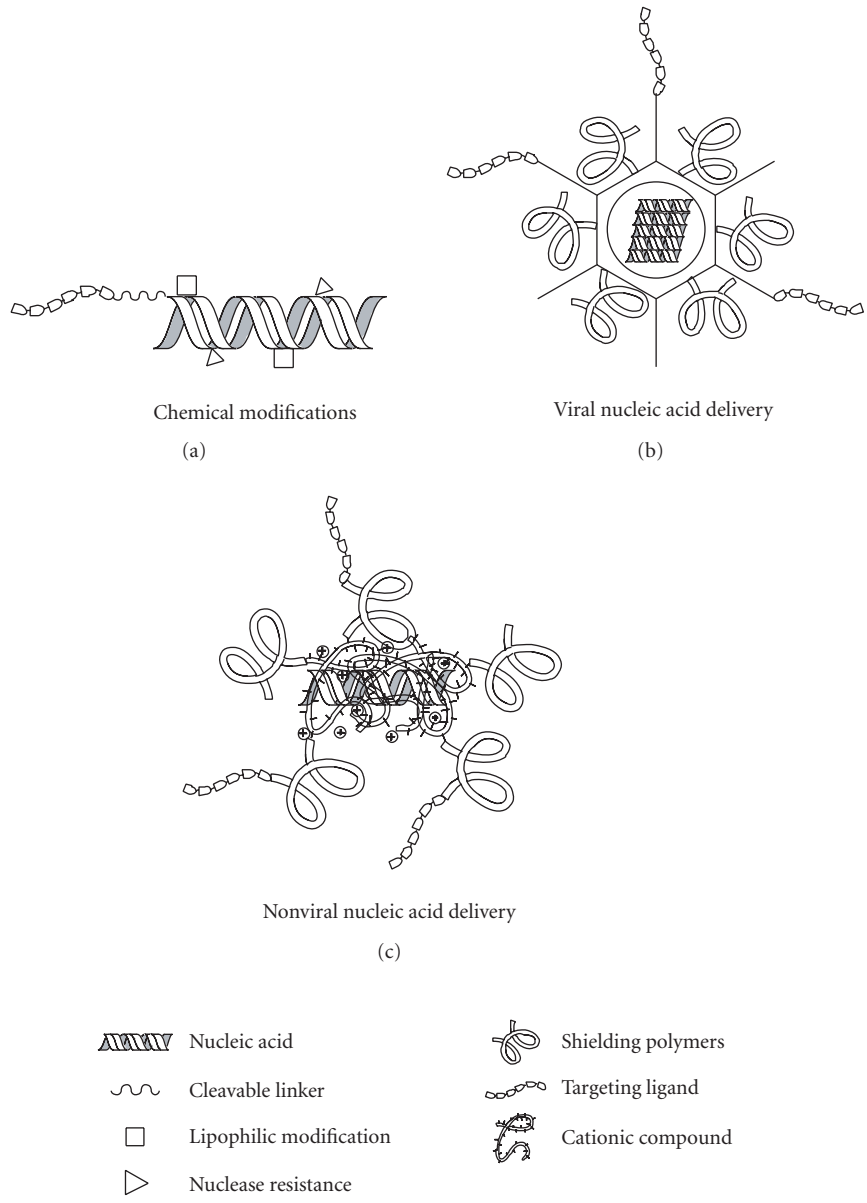

Figure 2: Strategies for siRNA delivery. Strategies are based on (a) chemical modifications of siRNA, (b) targeting of siRNA using viral vectors, or (c) nonviral delivery systems.

Synthetic nonviral delivery systems are a diverse class of molecules used in different nucleic acid delivery strategies that range from relatively simple cationic complexation for local administration to targeted shielded systems for intravenous injection. Their adaptability to specific targeting requirements is an important advantage, although optimization of delivery efficiency continues to remain important.

FINAL REMARKS

Over the last decades, research on the promises of nucleic acids for therapeutic intervention and the difficulties encountered in turning these promises into clinical reality has provided a clearer picture of the development steps that are needed to transform nucleic acids into actual drug molecules. As a result siRNA has been able to make a remarkable rapid progress from initial discovery as functional mediator of RNA interference in mammalian cells in 2001 to three clinical trials at the end of 2005: two in age-related macular degeneration, the other in respiratory syncytial virus infection [71]. Nevertheless, the choice of the diseases also reflects the delivery difficulties encountered for this class of nucleic acids. These diseases were selected partly because the target cell delivery problems are relatively low as these pathologies are confined to specific and accessible sites. To further improve target specificity, also in view of possible adverse effects occurring when siRNA is processed by nontarget cells, and to allow application of siRNA for systemic treatment several strategies can be proposed (Figure 2). Taken together they serve to increase nuclease resistance, to reduce renal

excretion/specific cell uptake, to promote uptake by the target cells, and to ensure correct intracellular trafficking to the site of action. As the first preclinical proofs of principle have been delivered showing therapeutic effects of locally and systemically delivered siRNAs, it is expected that these strategies will soon translate into viable clinical development programs.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 87340, Pages 1–13
DOI 10.1155/JBB/2006/87340

Review Article

RNA-Mediated Gene Silencing in Hematopoietic Cells

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Received 8 February 2006; Accepted 3 April 2006

In the past few years, the discovery of RNA-mediated gene silencing mechanisms, like RNA interference (RNAi), has revolutionized our understanding of eukaryotic gene expression. These mechanisms are activated by double-stranded RNA (dsRNA) and mediate gene silencing either by inducing the sequence-specific degradation of complementary mRNA or by inhibiting mRNA translation. RNAi now provides a powerful experimental tool to elucidate gene function in vitro and in vivo, thereby opening new exciting perspectives in the fields of molecular analysis and eventually therapy of several diseases such as infections and cancer. In hematology, numerous studies have described the successful application of RNAi to better define the role of oncogenic fusion proteins in leukemogenesis and to explore therapeutic approaches in hematological malignancies. In this review, we highlight recent advances and caveats relating to the application of this powerful new methodology to hematopoiesis.

INTRODUCTION

The discovery of RNAi

In 1998 Andrew Fire and Craig Mello discovered in a series of experiments in *Caenorhabditis elegans* that injection of sense or antisense RNAs led to negligible decreases of target RNA, whereas introduction of dsRNA resulted in effective and specific degradation of cytoplasmic mRNA. Furthermore, these silencing effects of dsRNA in *C elegans* were systemic and heritable [1]. Later on, the sequence-specific gene silencing capacity of dsRNA, now known as RNA interference (RNAi), has been linked to previously described gene silencing phenomena such as posttranscriptional gene silencing (PTGS), co-suppression in plants, and quelling in fungi [2–7]. Following this exciting and fundamental discovery, intense studies were undertaken with the purpose to dissect the molecular mechanism of RNAi. Indeed, in the past few years, many details of the biogenesis of small dsRNAs have been elucidated, and components, structure, and function of protein complexes of the RNAi machinery have been identified. RNAi has thus emerged as an evolutionarily highly conserved and fundamental mechanism for the regulation of gene expression and has rapidly been developed into a tool to analyze gene function.

RNAi molecular pathways

The detailed molecular mechanism of RNAi has been the subject of numerous recent reviews [8–16]. As this review concentrates on delivery of RNAi in the hematopoietic system and discusses its potential diagnostic and therapeutic applications in hematology, we will only briefly resume the principal phases of dsRNA-mediated gene silencing pathways as schematically represented in Figure 1.

Two types of small noncoding dsRNA can serve as effector molecules and trigger RNAi: small interfering RNA (siRNA) and micro RNA (miRNA). Long dsRNAs, introduced experimentally into the cell or generated as intermediates during viral infections [17] or as aberrant transcripts derived from inverted tandem repeats and transposons [18], are processed in the cytoplasm by a ribonuclease (RNase) III-like enzyme called Dicer [19, 20] into siRNA duplexes of 21–25 nt in length with 3′ dinucleotide overhangs, 5′-phosphates, and 3′-hydroxyl termini [21].

With the discovery of micro RNAs (miRNAs) the important physiological role of RNA-mediated gene silencing for regulating gene expression during development [22], differentiation [23], and apoptosis [9, 24], as well as its possible involvement in diseases like cancer, soon became manifest (see [8, 9] for review). miRNAs constitute a large class of endogenously expressed, highly conserved, noncoding small RNA molecules which act as negative regulators of gene expression in a variety of organisms ranging from plants to mammals [9]. Usually miRNAs are transcribed from endogenous genes by RNA polymerase II [25, 26] as long primary transcripts (pri-miRNAs) (see [27] for review) possessing 5′-caps and 3′-poly A tails [25, 28] (Figure 1). Recent studies

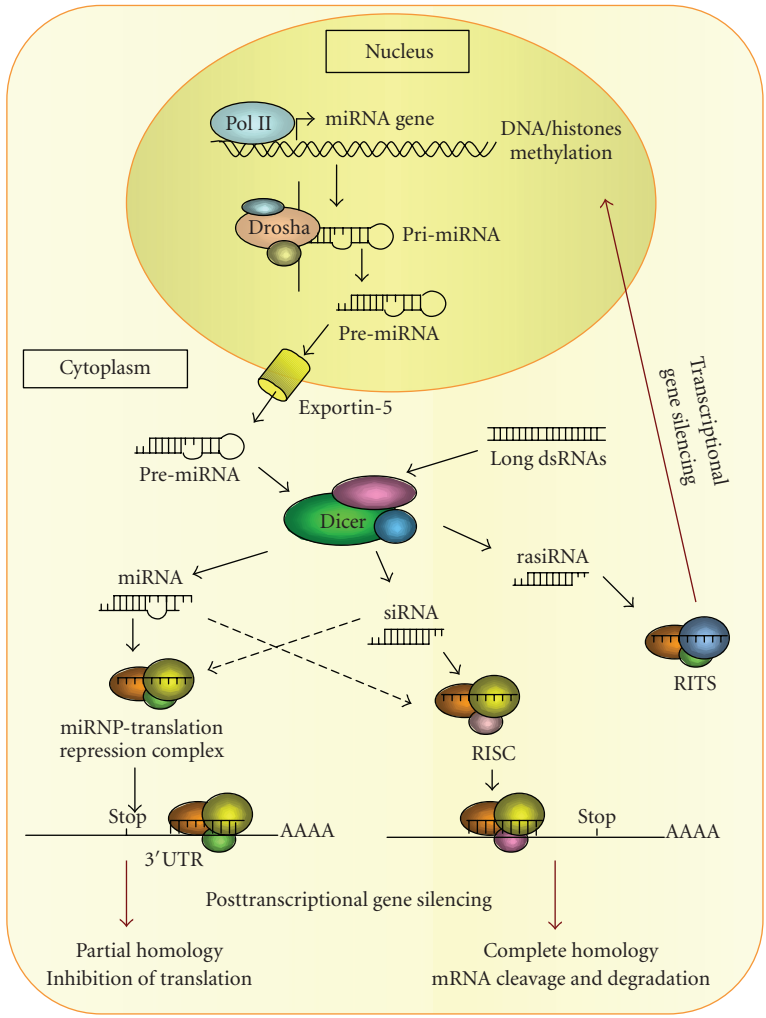

Figure 1: *Schematic representation of RNA-mediated gene silencing pathways.* miRNAs are transcribed from endogenous genes by RNA polymerase II as long primary transcripts (pri-miRNAs). In the nucleus, Drosha, an RNase III-like enzyme, releases the characteristic stem-loop structure of the ~ 70 bp precursor-miRNA from the primary transcript (pre-miRNA). The pre-miRNAs are then transported from the nucleus to the cytoplasm by exportin-5, where they are processed by Dicer to generate the mature miRNA of 21–23 nt in length. Similarily, siRNA duplexes of 21–25 nt in length are generated from long dsRNAs in the cytoplasm by Dicer as well. Only the antisense strand of the mature si/miRNAs is retained in the active ribonucleoprotein effector complexes (RISC or miRNP) and acts as a guide to target the homologous mRNA. Normally, siRNAs are fully complementary and induce the cleavage and degradation of their target mRNA transcript. In contrast, miRNAs usually bind only with partial complementarity to the 3′ untranslated region (UTR) of their cognate mRNAs and lead to translational inhibition. Depending on the cell context and the degree of complementarity between the small RNA effector and the mRNA target, both siRNA and miRNA can induce posttranscriptional gene silencing through degradation or translational inhibition. In addition, transcriptional gene repression has been described by repeat-associated siRNA (rasiRNA). They are believed to be loaded into RNAi-induced transcriptional gene silencing (RITS) complexes and may target homologous genomic loci. However, this mechanism is not yet fully understood.

suggest that miRNA expression is regulated at the level of transcription [29–31], similarly to mRNA and likely involving a similar or identical molecular machinery. The pri-miRNAs undergo a two-step processing to give rise to the mature miRNAs: in the nucleus, the characteristic stem-loop structure of the precursor-miRNA (pre-miRNA) of ~ 70 bp is released from the primary transcript by another RNase III-like enzyme called Drosha [32–36]. The pre-mRNAs are then transported from the nucleus to the cytoplasm by exportin-5 [37–40], where its 2 nt 3′-hydroxyl terminus is recognized by Dicer, which generates the mature miRNA of 21–23 nt length [41–43]. Dicer thus represents the core of the RNAi machinery where the different RNA-mediated gene silencing processes converge (Figure 1).

Only one strand of the mature si/miRNAs, distinguished by the lower thermodynamic stability at its 5′ terminus

[44, 45], is retained in the active ribonucleoprotein effector complexes and acts as a guide to target the homologous mRNA (Figure 1). Normally, siRNAs are fully complementary to their cognate mRNAs and guide the RNA-induced silencing complex (RISC) [46, 47] to the target transcripts, followed by cleavage and degradation. A member of the Argonaute (Ago) family of proteins [48, 49], which contain a small RNA-binding domain (PAZ [50]) and an RNase activity (PIWI domain [51]), constitutes the catalytic ribonuclease component of RISC responsible for cleaving the target mRNA at the center of the region complementary to the guide RNA [48, 49]. RISC is then released and the cleaved mRNA is degraded by cellular exonucleases. Except in plants where they have perfect homology to their targets and act exactly as siRNAs inducing mRNA degradation [52] upon integration into complexes usually referred to as miRNA-ribonucleoproteins (miRNPs), most miRNAs bind only with partial complementarity to the 3′ untranslated region (UTR) and lead to translational inhibition downstream of the initiation step [53]. In fact, apart from the distinction based on their biogenesis, a precise boundary between si- and miRNA functionality cannot always be established, since both have been shown to be capable to target mRNA for degradation or translational inhibition, depending on the cellular context and the degree of complementarity between the RNA effector and the mRNA target [46, 54–58].

In addition to posttranscriptional gene silencing, RNAi has also been demonstrated to induce transcriptional repression through RNA-directed DNA methylation in plants [59], and recent studies have reported similar effects even in human cells [60, 61], inducing heterochromatin formation (see [62, 63] for review).

RNAi IN MAMMALIAN CELLS: DESIGN, DELIVERY, AND TARGET VALIDATION

After its discovery, RNAi was rapidly employed as a powerful tool for large-scale reverse genetic screens in *C elegans*, where it can be easily induced by direct injection of dsRNAs, by feeding worms with bacteria engineered to express siRNAs, or simply by soaking the animals in medium containing siRNAs. Furthermore, in nematodes, RNAi acts systemically and is a long-lasting heritable event due to the presence of RNA-dependent RNA polymerases (RdRPs) which allow the amplification of the trigger and the perpetuation of the silencing process [64–66].

In mammals, however, initial efforts to use RNAi for specific gene silencing encountered more difficulties, especially due to the induction of nonspecific inhibition of gene expression resulting from the activation of the interferon (IFN) response pathway by dsRNAs longer than 30 bp [67]. The discovery from Elbashir et al [68] and Caplen et al [69] that RNA duplexes of 21 nt in length, mimicking the Dicer cleavage products, were able to mediate efficient and specific RNAi upon transfection into mammalian cells without eliciting the INF response, finally enabled loss-of-function studies of specific target genes in mammalian systems as well.

Designing RNAi effector molecules

To design efficient siRNAs several parameters should be considered. Although duplex RNAs of 21 nt in length were shown to be the most effective RNAi triggers, recent studies suggest that 27- to 29-mers may be more active than 21-mers [70–72]. This could be due to the fact that these dsRNAs are processed by Dicer and thus may be incorporated directly and more efficiently into RISC [73]. Reynolds et al [74] conducted systematic analyses to evaluate physicochemical characteristics associated with highly functional siRNAs and set up an algorithm with several criteria which significantly improved selection of potent siRNAs. To comply with the rules promoting asymmetric incorporation into RISC, the base pair at the 5′ end of the siRNA antisense (guide) strand should have a lower thermodynamic stability compared with the 3′-end [44, 45]. Accordingly, the presence of three or more A/U nucleotides at the 3′-terminus of the sense strand was defined as a criterion for siRNA functionality [74]. Low G-C content (30%–52%), lack of internal inverted repeats which can form secondary structures, and specific nucleotide preferences at positions 3 (A), 10 (U), 13 (absence of G), and 19 (A, absence of G or C) on the sense strand also increased the probability of selecting a potent siRNA in this study [74]. Concerning the specificity of the sequence match necessary to achieve efficient gene silencing, different groups have reported varying degrees of mismatch tolerance for siRNA-mediated silencing [75–81]. Mismatched small RNAs may still be competent and can function like miRNAs which may bind to mRNAs with multiple target sites in the 3′-UTR [82]. miRNA binding is not limited to the 3′-UTR but can include the coding sequence of an mRNA as well [83]. These results demonstrate the possibility of off-target effects by siRNAs used for experimental or therapeutic purposes. Therefore, for prevention of cross-reactive silencing, a BLAST search of potential target sequences should be performed in order to exclude candidate siRNAs with some degree of homology with other genes [74, 84]. For the efficacy of gene silencing, the overall stability of the antisense RNA/RISC-mRNA complex is probably more important than the absolute number of mismatches. Accordingly, base pairing at the center of the duplex is critical, while one or two mismatches located at the 3′- or 5′-end of the siRNA may be well tolerated [85]. Since regions which are not involved in intramolecular folding have been demonstrated to be optimal targets [86], computational approaches have been reported to analyze the secondary structure and the local folding of the target mRNA. However, all predictions based on physicochemical characteristics, sequence homologies, or secondary structure can not guarantee the generation of an efficient siRNA, and functional testing is still always required. Similarly, different siRNAs targeting the same gene may have different silencing efficacies not always predictable by the parameters discussed above. Therefore, more than one target sequence should be tested empirically to identify the optimal small RNA for efficient and specific silencing of a given target mRNA.

RNAi delivery in mammalian cells

RNAi in mammalian cells can be triggered by direct introduction through injection, electroporation, lipid-mediated transfection, nanoparticles, or antibody bound enzymatically generated or chemically synthesized siRNAs, among others. Alternatively, siRNAs or small hairpin RNAs (shRNAs) can be delivered by vector-based intracellular expression.

Synthetic siRNA-mediated RNAi

siRNAs can be synthesized chemically [75, 87, 88], generated enzymatically through in vitro transcription by T7 phage polymerase [89, 90], or through endonuclease digestion by recombinant Dicer of in vitro transcribed long dsRNA [91, 92]. In mammalian cells, direct delivery of siRNAs can only induce transient silencing due to their limited half-life and to their dilution during cell division.

Vector-based RNAi

Transient downregulation of gene expression may not be sufficient for many applications, for example, for studies of proteins with long half-lives in rapidly dividing cells. In order to produce long-lasting RNAi in mammalian cells, plasmids and viral expression vectors have been developed to drive continuous intracellular expression of siRNA or shRNAs under the control of highly active RNA polymerase III promoters such as U6 or H1 [93, 94].

As represented in Figure 2, the two strands of an siRNA can be transcribed from distinct expression units, either cloned in tandem or in two separate vectors [95, 96], or can result from bidirectional transcription of a single 19- to 29-mer DNA fragment under the control of two opposite promoters [97, 98]. The intracellular expression and hybridization of the two strands gives rise to functionally active siRNA duplexes. However, the most commonly used approach involves the intracellular expression of shRNAs. They are transcribed as single-stranded RNAs from an expression cassette inserted immediately downstream of the pol III promoter, which contains, in the following order, a 19 nt sequence homologous to the target mRNA, a spacer 6 to 9 nucleotides in length, the antisense sequence, and the RNA pol III terminator signal composed of a stretch of about 6 thymidines. After transcription, the resulting stem-loop RNA structure, like miRNA precursors, is cleaved by Dicer to yield a functionally active siRNA [93, 99, 100] (Figure 2). A second generation of RNAi-delivering vectors is based on RNA pol II promoters driving transcription of shRNAs incorporated into a miRNA chimeric transcript, comprising flanking sequences optimized for Drosha/Dicer processing [100, 101].

A major advantage of vector-dependent RNAi is the usage of selectable markers to generate stable transfectants or of reporter genes such as green fluorescent protein (GFP) or red fluorescent protein (RFP) to identify and eventually isolate the si/shRNA expressing cells in a quantitative manner. When using shRNA-expressing vectors, however, it is absolutely necessary to confirm the sequence of the shRNA expression cassette, because genetic recombination and/or introduction of point mutations are very frequent and can occur in almost every step of the cloning strategy.

Finally, when the target gene to be silenced is essential for cell survival and/or proliferation and the constitutive knockdown of its expression is even lethal, employing inducible or tissue-specific RNAi could be especially important [100]. In the last years, various methods for inducible expression of shRNAs have been described. Tetracycline-inducible [94, 102, 103] and ecdysone-inducible [104] expression systems have been reported which mediate induced and reversible downregulation of gene expression. However, no standard technique for inducible RNAi has been established so far.

Many suspension and primary cells are difficult to transfect efficiently. However, viral transduction strategies allow stable induction of RNAi in these cells. Particularly, the capacity of lentiviruses to integrate into the genome of noncycling cells, such as stem cells or terminally differentiated cells, renders lentiviral vectors much more efficient than retroviral vectors in inducing RNAi in these cells [105, 106].

Finally, transgenic technology has also been adapted for in vivo delivered RNAi in mice. Transgenic animals have been reported which produce siRNAs constitutively [107] or conditionally, in a stage- or tissue-specific manner [108], to repress selected target genes. Tiscornia et al [106] efficiently used a lentiviral vector system to express siRNA in preimplantation mouse embryos.

In vivo systemic delivery of siRNAs

Chemical modifications are required to potentiate siRNA nuclease and thermodynamic stability in vivo without compromising their efficacy. Recently, several groups reported different approaches for systemic in vivo delivery of siRNAs. Soutschek et al [109] described intravenous injection in mice of chemically modified naked siRNAs coupled to a cholesterol group chemically linked to the terminal hydroxyl group of the sense strand to promote entry into the cells. In vivo delivery of chemically modified siRNAs encapsulated into liposome particles has been recently reported by Morrisey et al [110], and Song et al [111] described an antibody-based delivery system which could offer a possibility for systemic, cell-type-specific siRNA delivery.

Specificity of RNAi-induced gene silencing

Prior to functional analysis, monitoring the level of target mRNA expression is necessary for siRNA target validation. Quantitative RT-PCR is a fast and reliable method to measure target transcript levels in specific versus control siRNA-treated cells, to ascertain that any phenotypic changes are really due to specific gene knockdown and not to nonspecific effects of the RNAi strategy. This issue can be further addressed by verifying that the same phenotype is induced by siRNAs homologous to different regions of the target transcript. The correlation between the extent of gene silencing and dose of the delivered si/shRNA can offer some evidence of specificity. Finally, the availability of a rescue experiment

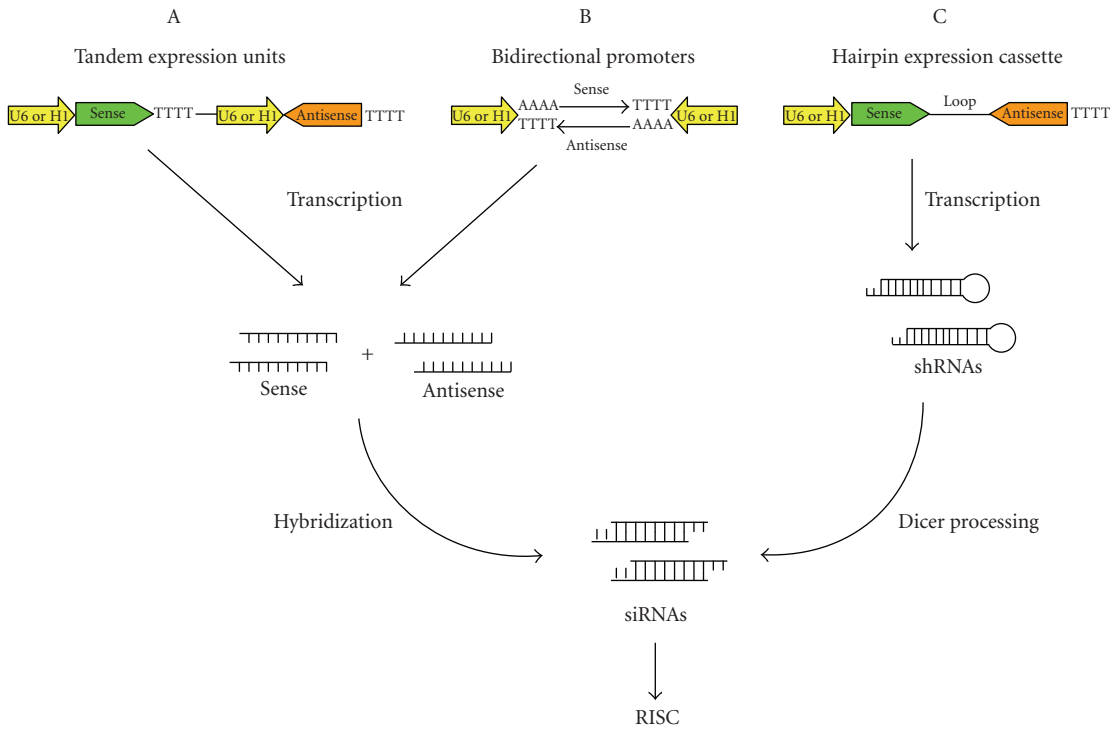

Figure 2: *Schematic representation of vector-based RNAi*. The two strands of an siRNA can be transcribed from two tandem expression units where the expression of the sense and the antisense strands is driven separately by their own respective promoter (A), or from a single DNA fragment under the control of two opposite promoters (B). After intracellular expression, hybridization of the two strands gives rise to functional siRNA duplexes. shRNAs are transcribed as single-stranded RNAs from a hairpin expression cassette cloned immediately downstream of an RNA pol III promoter containing the sense strand homologue to the target of interest followed by a 4 to 9 nt spacer, the antisense strand, and the terminator signal. After transcription the resultant stem-loop structure is cleaved by Dicer and yields a functional siRNA (C).

able to revert the loss-of-function phenotype can ensure RNAi specificity.

RNAi IN THE HEMATOPOIETIC SYSTEM

siRNAs and hematopoietic cells

Apart from its transient nature in mammals, the use of RNAi in primary hematopoietic cells is limited by the difficulty to deliver siRNA through conventional transfection methods. In contrast, lentiviruses have been shown to efficiently transduce human hematopoietic stem and NOD/SCID repopulating cells (HSCs and SRCs) as well as more committed colony forming progenitors [112–114] and can offer a useful means for effective and stable delivery of RNAi triggers in the hematopoietic system. Lentiviral transduction was successfully employed by our group to induce RNAi against the common β chain of the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5 in human $CD34^+$ SRCs and colony forming cells [115]. Similarly, Schomber et al [116] reported efficient and stable silencing of the p53 gene in human cord blood-derived $CD34^+$ cells through lentivirus-mediated RNAi.

Application of siRNAs to target leukemia-associated oncogenes

Chromosomal translocations leading to the expression of chimeric oncoproteins are frequently involved in malignant transformation in leukemias and lymphomas. In the past few years, RNAi technology has been used to specifically silence the expression of translocation products (Table 1). For example, the bcr-abl oncogene resulting from the $t(9;22)$ translocation characterizes chronic myelogenous leukemia (CML) and some variants of acute lymphoblastic leukemia (ALL). It codes for a constitutively active cytoplasmatic tyrosine kinase which is both necessary and sufficient for leukemic transformation in several models [129–131]. Different research groups [117–120] have demonstrated the feasibility of specific interference with the bcr-abl expression without affecting the expression of wild-type c-abl or c-bcr using breakpoint-specific siRNAs delivered by electroporation in bcr-abl positive hematopoietic cell lines and primary $CD34^+$ cells from CML patients. Upon siRNA treatment, inhibition of survival and proliferation, increased sensitivity to the abl-specific tyrosine kinase inhibitor imatinib mesylate (STI571) [123, 132], and increased apoptosis were observed [119, 120].

Table 1: RNAi targeting hematopoietic fusion genes.

Oncogene target	Disease implication	Reference
BCR-ABL	Chronic myeloid leukemia	[117]
		[118]
		[119]
		[120]
		[121]
		[122]
AML1-MTG8	Acute myeloid leukemia	[123]
TEL-PDGFβR	Chronic myelomonocytic leukemia	[124]
MLL-AF4	Acute lymphatic leukemia	[125]
NPM1-ALK	Anaplastic large-cell lymphoma	[126]
		[127]
FLT3-ITD	Acute myeloid leukemia	[128]

In a recent study, our group used lentiviral gene transfer of shRNAs to trigger stable RNAi targeting the bcr-abl oncogene [133]. Stable, but not transient, RNAi was demonstrated to induce depletion of bcr-abl positive cells from suspension cultures. This depletion, as well as the degree of bcr-abl gene silencing, correlates with the multiplicity of lentiviral infection (MOI), the number of lentiviral integration into the host cell genome, and the expression level of the RFP reporter gene: cells with lower RFP expression and fewer lentiviral integrations could survive and were selected in suspension cultures but still showed reduced bcr-abl expression, aberrant proliferation kinetics, and enhanced sensitivity to STI571 as compared to controls. Furthermore, in contrast to transient RNAi [118], stable RNAi-induced silencing of bcr-abl inhibited the colony forming capacity of primary $CD34^+$ cells from CML patients.

Heidenreich et al [123] used siRNAs to specifically repress the AML1/MTG8 fusion product resulting from the $t(8;21)$ translocation [134] found in about 10% to 15% of all cases of de novo AML. Electroporation of siRNAs specific for the fusion site of the AML1/MTG8 into the Kasumi-1 $t(8;21)$-positive cell line specifically suppressed the expression of the fusion product, without impairing the expression of the two respective wild-type genes. Despite the transient character of the RNAi strategy employed, the authors described some functional effects due to the suppression of the fusion protein such as increased myeloid differentiation and reduced clonogenic potential upon TGFβ/vitamin D treatment.

Stable retroviral delivery of shRNAs was used by Chen et al [124] to target the fusion sequence of the TEL-PDGFβR fusion product derived from the $t(5;12)$ translocation, a recurrent cytogenetic aberration associated with chronic myelomonocytic leukemia (CMML) [135]. Stable RNAi-mediated inhibition of TEL-PDGFβR significantly reduced the proliferation of TEL-PDGFβR-transformed Ba/F3 cells, but did not restore IL-3 dependence, concordant with a marked decrease, but not abrogation of TEL-PDGFβR expression and selection of TEL-PDGFβR expressing cells. The authors also reported a significantly prolonged disease latency and survival of nude mice or Balb/C mice injected with TEL-PDGFβR-transormed Ba/F3 cells coexpressing siRNA as compared with injection of TEL-PDGFβR-transformed cells not expressing siRNA. However, as observed in cell culture, the expression of siRNA alone was not sufficient to completely abrogate TEL-PDGFβR-induced transformation in these murine models. A synergistic effect between siRNAs and small molecule inhibitors of tyrosine kinase activity, such as imatinib, was also demonstrated in TEL-PDGFβR-transformed Ba/F3 cells.

The mixed-lineage leukemia (MLL) gene is involved in numerous translocations in a variety of leukemias [136]. Most frequently, the MLL gene is fused to the AF4 gene as a consequence of the $t(4;11)$ translocation [137–139], found in acute lymphoblastic leukemia (ALL) with poor prognosis in infants [140]. Thomas et al [125] applied siRNAs to silence MLL-AF4 and demonstrated decreased proliferation and clonogenicity of $t(4;11)$-positive leukemic cells as well as induction of apoptosis through caspase-3 activation and repression of the BCL-X_L anti-apoptotic gene. They also observed that MLL-AF4 depletion resulted in a reduced expression of the homeotic genes HoxA9, MEIS1, and HoxA7, known to be upregulated by MLL fusion proteins, and of the CD133 marker for hematopoietic stem cell and early progenitors, which may suggest a reactivation of hematopoietic differentiation. Finally, using a NOD/SCID mouse xenotransplantation model, the authors showed that siRNA-mediated repression of MLL-AF4 compromised leukemic engraftment and the development of leukemia in vivo.

The $t(2;5)$ chromosome translocation fuses the ALK (anaplastic lymphoma kinase) gene on chromosome 2 to the nucleophosmin (NPM1) gene on chromosome 5 and is associated with anaplastic large-cell lymphomas (ALCLs) [141]. In a recent study, Piva et al [127] reported that silencing of NPM1-ALK induced by shRNAs directed against the 3′ sequences encoding the cytoplasmatic domain of ALK-R,

which is retained in all oncogenic fusion proteins involving ALK, leads to abrogation of NPM1-ALK-mediated transformation of MEF cells and inhibition of cell growth in several human NPM1-ALK-positive cell lines. Moreover, an increased number of apoptotic cells together with caspase activation and downregulation of the anti-apoptotic protein survivin were detected in ALCL cells 4 days after lentivirus-mediated RNAi. Similar results were seen in vivo: shRNA-expressing ALCL cells injected into NOD/SCID mice revealed a reduction in tumor formation as compared to control cells. Furthermore, in a second series of experiments, injection of lentiviruses driving shRNA expression directly into ALCL tumor masses showed growth inhibition of neoplastic cells, and histologic sections of the tumors demonstrated the presence of large necrotic regions and, in areas with retained viability, many apoptotic cells.

The receptor FMS-like tyrosine kinase 3 (FLT3) is the single most frequently mutated gene in AML. It is constitutively activated by internal tandem duplications (ITDs) within the juxtamembrane domain or by point mutations within the catalytic kinase domain in approximately 30% of AML patients [142, 143] and appears to confer an unfavourable prognosis. RNAi-mediated silencing of FLT3 was reported by Walters et al [128]. The authors used an siRNA pool to effectively downregulate the expression of FLT3 in FLT3-ITD-positive human leukemia cells and showed diminished phosphorylation of downstream signalling molecules, comprising STAT5, MAPK, and Akt, inhibition of cell proliferation, and induction of apoptosis. In addition, upon siRNA treatment in these cells, they found increased sensitivity to treatment with the FLT3 inhibitor MLN518, further demonstrating the potential benefit of such combined therapeutic approaches.

miRNAs in the hematopoietic system

miRNAs associated with hematopoietic differentiation

Fine modulation of gene expression is essential for the correct realization of differentiation programs. Consistent with this, several groups recently demonstrated the implication of miRNAs in controlling hematopoietic differentiation.

Chen et al [23] described three miRNAs, miR-181, miR-223, and miR-142s, which are differentially expressed in the murine hematopoietic system, and showed that miR-181 plays a specific role in B-cell differentiation. They found that miR-181 is normally expressed at low levels in murine hematopoietic progenitors and becomes upregulated during B-cell differentiation. Overexpression of miR-181 in hematopoietic progenitors gives rise to a greater fraction of B-lymphoid cells than in wild-type progenitors, in vitro as well as in vivo.

In a recent publication, Felli et al [144] described miR-221 and miR-222 as inhibitors of normal erythropoiesis and indicated the kit receptor mRNA as a major target of these two miRNAs. Using microarray chip and Northern blot-analysis, they showed that miR-221 and miR-222 are downregulated in erythropoietic cultures of cord blood $CD34^+$ progenitors. In addition, they observed an impairment of human $CD34^+$ cell engraftment in NOD/SCID mice as well as an inhibition of cell growth in the c-kit+ TF-1 erythroleukemic cell line upon overexpression of miR-221 and miR-222.

Recent studies conducted by Fazi et al [29] revealed the implication of miR-223 in human myeloid differentiation: miR-223 expression increases during retinoic acid- (RA-) induced granulocytic differentiation of the NB4 promyelocytic cell line as well as of blasts from patients with acute promyelocytic leukemia (APL) undergoing RA treatment. The authors depicted a finely regulated network involving miR-223 and the transcription factors C/EBPα, well known for its implication in granulocytic differentiation [145–147], and NFI-A [148, 149]. C/EBPα and NFI-A, which can induce or repress miR-223 expression, respectively, are in competition for an overlapping binding site on the miR-223 promoter. In undifferentiated cells, NFI-A maintains miR-223 and consequently its translation inhibitory effect at low levels. Upon RA treatment, C/EBPα displaces NFI-A from the miR-223 promoter, thus activating its expression. Interestingly, NFI-A is a target of miR-223 which, through a positive feedback, represses NFI-A translation, reduces the competition with C/EBPα, and maintains sustained levels of its own expression.

miRNA alterations in hematological malignancies

The expression of about one-third of human mRNAs appears to be regulated by miRNAs, each of which, according to computational analysis, is predicted to regulate a broad spectrum of different mRNAs [150], revealing a very complex regulatory network. As cancers essentially derive from alteration of gene expression and/or gene function, it is not surprising that several recent publications supported the direct involvement of miRNAs in tumorigenesis. Approximately 50 percent of the known human miRNA genes are located at fragile sites and cancer-associated regions of the genome [151]. Dysregulation of various human miRNAs has been associated with leukemias and lymphomas: the precursor of miR-155 was found to be overexpressed in the majority of childhood Burkitt lymphoma [152]; the miR-15a/miR-16 cluster at locus 13q14 is frequently deleted or downregulated in patients with B-cell chronic lymphocytic leukemia (CLL), mantle cell lymphoma, and multiple myeloma [153]. As miR-15/16 was demonstrated to induce apoptosis by targeting the apoptosis inhibitor protein BCL2 in CLL cells [154], downregulation of miR-15a and miR-16-1 can contribute to malignant transformation through BCL2 upregulation and inhibition of apoptosis. The miR-17-92 polycistron, located at 13q31, is amplified in human B-cell lymphomas [155]. He et al [156] found that enforced expression of the miR-17-92 cluster can augment the oncogenic potential of *c-myc* in a mouse B-cell lymphoma model, offering the first evidence of a miRNA to act as an oncogene. O'Donnell et al [30] further confirmed the relationship between this miR cluster and cancer. Using a lymphoma cell line with inducible *c-myc* expression, they demonstrated that the miR-17 cluster is specifically and directly upregulated by *c-myc*, but at least miR-17-5p and

miR-20a downregulate E2F1-protein expression, a target of *c-myc* which promotes cell cycle progression. These findings reveal a feedback mechanism through which c-Myc activates E2F1 transcription and simultaneously induces inhibition of its translation.

Recent microarray-based studies have provided evidence that specific alterations in human miRNA expression profiles are associated with specific types of cancers. Lu et al [157] established a sensitive method to analyze the expression profiles of 217 miRNAs in a panel of 334 samples representing diverse human normal tissues and corresponding tumors. They observed a general downregulation of miRNAs in tumors compared with normal tissues and demonstrated that miRNA expression profiles correlate with the developmental origins of specific cancers. Furthermore, even within a single developmental lineage, distinct patterns of miRNA expression seem to reflect the mechanism of transformation. Indeed, clustering of miRNA profiles of bone marrow samples from patients with acute lymphoblastic leukemia (ALL) showed a nonrandom distribution into three major groups in correlation with previously characterized molecular alterations and phenotypic classifications (BCR-ABL-positive and TEL-AML1-positive samples, T-cell acute lymphoblastic leukemias, and mixed lineage leukemias). Their results suggest that miRNA expression profiles could be more accurate for the classification and diagnosis of human cancers than mRNA microarrays.

Using miRNA profiling, Calin et al [158] found that different patterns of miRNA expression distinguish CLL cells from normal CD5+ B cells. In a recent study based on genome-wide expression profiling of a large number of samples from CLL patients [159], the same group showed that a miRNA signature is associated with the presence of other known prognostic factors (levels of ZAP-70 expression and the mutational status of the immunoglobulin heavy-chain (IgV_H) gene) and with disease progression in CLL: a molecular signature composed of 13 miRNAs differentiated CLL patients with high levels of ZAP-70 expression and unmutated IgV_H from patients with low ZAP-70 expression and mutated IgV_H. Nine of these miRNAs were significantly overexpressed in the first group of patients, associated with a poor prognosis. Furthermore, 9 miRNAs of the prognostic signature were able to discriminate between patients with a short interval from diagnosis to therapy and patients with a longer interval: in the first group, 8 of the 9 miRNAs were upregulated, suggesting their involvement in disease progression. The authors also showed some functionally relevant mutations in miRNA genes in CLL. Some of these mutations are located in the flanking sequences of the pre-miRNA, compromising the correct processing and expression of the mature miRNA.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite many remaining technical problems, current advances in strategies to extend genome-wide screens with siRNA or shRNA libraries to mammalian cells [160–167] as well as specific gene silencing approaches may finally facilitate the identification of essential genes involved in human diseases and may identify new potential therapeutic targets. On the other hand, disease- and stage-specific systematic analysis of miRNA gene-expression profiles may help to establish new diagnostic and prognostic markers.

Employing RNAi in a therapeutic setting may still encounter numerous obstacles: the issue of efficient delivery in a clinical setting, as well as problems deriving from toxicity, and possible off-target effects.

While viral delivery systems are certainly of great utility for experimental models, further studies are necessary before their possible therapeutic application may become possible in the future.

If all these obstacles can be overcome, cancer-specific oncogenes, such as the fusion genes produced by chromosomal translocations involved in several types of leukemia, could be suitable candidates for tumor cell-specific targeting in RNAi-mediated therapeutic approaches. Beside those, gene expression by infectious organisms may be targeted by RNAi. Indeed, early clinical trials are under way or being started targeting vascular epidermal growth factor (VEGF) receptor in age-related macular degeneration or genes expressed by respiratory syncytial virus (RSV). Finally, as suggested by recent reports demonstrating the cooperative effects of RNAi and selective molecular inhibitors, such as imatinib for Bcr-Abl [119, 120] and TEL-PDGFβR [124], and the kinase inhibitor MLN518 for FLT3 [128], combination of targeted therapies including small molecules and RNAi could be taken into consideration, especially when drug resistance becomes a problem.

ACKNOWLEDGMENTS

We thank Michael Morgan and Michael Stadler for critical reading of the manuscript. M. Eder and M. Scherr are supported by the H & W Hector-Stiftung, the Wilhelm Sanders-Stiftung, and the Deutsche Forschungsgemeinschaft (SFB566).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 18657, Pages 1–12
DOI 10.1155/JBB/2006/18657

Review Article

Transplacental RNAi: Deciphering Gene Function in the Postimplantation-Staged Embryo

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Received 1 February 2006; Revised 26 April 2006; Accepted 2 May 2006

RNAi offers the opportunity to examine the role in postimplantation development of genes that cause preimplantation lethality and to create allelic series of targeted embryos. We have delivered constituitively expressed short hairpin (sh) RNAs to pregnant mice during the early postimplantation period of development and observed gene knockdown and defects that phenocopy the null embryo. We have silenced genes that have not yet been "knocked out" in the mouse (*geminin* and *Wnt8b*), those required during earlier cleavage stages of development (*nanog*), and genes required at implantation (*Bmp4*, *Bmp7*) singly and in combination (*Bmp4* + *Bmp7*), and obtained unique phenotypes. We have also determined a role in postimplantation development of two transcripts identified in a differential display RT-PCR screen of genes induced in ES cells by noggin exposure, *Aggf1* and an Est (GenBank AK008955). Systemic delivery of shRNAs provides a valuable approach to gene silencing in the embryo.

INTRODUCTION

With the sequencing of the mouse genome [1], there has been tremendous interest in teasing out the function of "every" gene. In the mouse, gene targeting using homologous recombination in embryonic stem cells (ESC) has provided a unique opportunity to probe gene function in development [2], and a number of powerful techniques have been developed to target genes in temporal or tissue specific ways. Unfortunately, these are time consuming and often require the development of multiple strains of mice, which then must be mated to obtain the desired cell-type specific gene targeting. The recent application of posttranscriptional gene silencing using RNA interference (RNAi) to silence target genes has been an efficient way to study gene function initially in *C elegans* and plants, later in mammalian cells in culture, and recently in embryos.

RNAi is a powerful alternative to traditional gene targeting using homologous recombination in ES cells, large scale mutagenesis, ribozymes, morpholinos, oligonucleotides, and so forth, for many reasons. Among these are simplicity in the design of the targeting construct, efficiency, and high throughput (reviewed in [3]). In addition, RNAi offers the ability to target specific exons/specific sequences within a gene [4], to study gene dosage phenotypes, to target multiple (redundant) genes, to target multiple members of a regulatory pathway, and to produce graded levels of knockdown analogous to allelic series, which is particularly useful in analyzing the effects of genes that have "threshold" effects rather than acting as binary on-off switches. In addition, RNAi may be particularly useful to avoid the confounding genetic background effects common to gene targeting using the limited number of "germ line" ESC lines, and finally, many other species (eg, rat) can be employed.

Relatively few studies have employed RNAi to study gene function in the developing embryo. RNAi has been electroporated [5, 6] or microinjected into oocytes or early zygotes [7–11], siRNA-transfected ES cells have been used to create germ line transgenic RNAi mice [12], or all ES embryos have been generated using tetraploid aggregation of RNAi-targeted ESC [13]. Delivery, particularly to postimplantation-staged embryos, continues to be a major limitation in the widespread application of this important technology.

Information regarding the prenatal delivery of plasmid DNA (pDNA) comes largely from the gene therapy field where in utero gene targeting/therapy has been proposed as a method to treat diseases that affect the developing embryo [14], which may ultimately be the most effective means to treat genetic defects. Various routes of pDNA delivery have been attempted for fetal "gene therapy" including direct injection of the fetus [15–17], injection into the placenta or umbilical cord [18, 19], injection into the amniotic cavity [20, 21], or the yolk sac [21],

typically resulting in the limited transduction of the embryo.

Intravascular delivery of naked DNA is increasingly recognized as a preferred route to deliver nucleic acids to target tissues [22] because of its simplicity and effectiveness and because high levels of transgene expression can be achieved and sustained (eg, [23]). However, it has required either high-pressure delivery to produce extravasation [24] or a tourniquet to keep the pDNA in place [23]. Tail vein injection has been employed to silence genes in neonatal [24], and adult mice [25–28]. Based on these reports, we have recently delivered shRNAs to pregnant mice and have observed gene silencing and additional six genes that play important roles in organogenesis of the early embryo.

MATERIALS AND METHODS

Development of targeting constructs

We developed a targeting construct that would allow us to deliver a single plasmid containing a small hairpin RNA (driven by the constitutively active H1 or U6 promoter) and a fluorochrome reporter driven by the CMV promoter (Figure 1). The vector backbone is the pCS2 plasmid (from David Turner), which contains two multiple cloning sites (MCS) for insertion of a DsRED and shRNA cassettes. A BamHI/XbaI fragment that contains the entire DsRed coding region was removed from pDsRed2-1 (Clontech) and ligated downstream of the CMV promoter in the first MCS. The H1 (GenBank AF191547) or the U6 (GenBank X06980) promoter was amplified in PCR with specific primers and SV129 mouse genomic DNA was then ligated into the second MCS. Gene-specific shRNAs were designed to target *Aggf1* (BC052410), *Bmp4* (GenBank X56848), *Bmp7* (NM007557), *geminin* (AF068780), *nanog* (AY278951), *Wnt8b* (NM011720), and Est1 (AK008955). Each shRNA is a ligated downstream of the H1 or U6 promoter to yield the final expression plasmid. All sequences are included in the supplemental data.

In addition to confirming that the plasmid reached the embryonic compartment (DsRed fluorescence), controls include empty plasmid (pRed) and hairpins containing three nucleotide substitutions (scrambled hairpins) that correspond to no known mRNA. Blast analysis confirms unique targeting of the hairpin and that no genes are targeted by the scrambled hairpin. We monitor target gene expression using PCR and at the protein level by Western blot or immunohistochemistry when an antibody is available. It is also important to monitor additional members of the signaling pathway, compensatory genes, irrelevant genes, and genes downstream of the target. We also monitor the interferon response gene *Oas1* (GenBank AF466823) [29] to determine if our construct elicits a nonspecific response.

Tail vein injections

These are carried out in mice as we have described previously [30]. Pregnant females or neonates are placed in a conical tube (open at the tip for air flow). A small hole is also drilled into the cap to accommodate the tail. Mice are warmed for 5 minutes using a heat lamp and heating pad, then shRNA expression plasmids (10 μg) diluted in Ringer's solution are injected into the tail vein. We use a 23-G needle and a volume of 200–300 μL using a slow steady pressure, usually over 10–20 seconds for pregnant mice.

Because research in our laboratory has focused on the early postimplantation period of development, we have typically delivered targeting constructs at E6.5 and autopsied embryos 24 h to 72 h later. We have also carried out limited studies at midgestation when the placental barrier is most robust, as well as on E17.5 when the barrier thins and delivery should be more complete. We examine the extent of DsRed expression in all embryos using epifluorescence, followed by scanning electron microscopy (SEM), sectioning, immunohistochemistry, Western blotting, and/or PCR. DsRed is typically expressed throughout the early embryo, without a preference for a particular tissue type.

Tissue analysis

Pregnant females and neonatal mice are sacrificed by cervical dislocation followed by rapid dissection of embryos and tissues. Embryos are dissected from the decidua and images are captured using a Leitz-inverted fluorescence microscope to determine the extent of DsRed expression. Embryos are then either embedded in OCT for frozen sectioning or placed in Trizol for RNA/protein extraction. For SEM or whole mount immunohistochemistry (IHC), embryos are fixed in 1% glutaraldehyde (SEM) or 2% paraformaldehyde (IHC), then stored at 4° prior to additional processing.

For scanning electron microscopy, embryos are dehydrated through graded alcohols, washed twice in hexamethyl disilazane (HMDS), oriented on SEM stubs, and sputter-coated with gold palladium. They are viewed and photographed using an Amray 1910 scanning electron microscope.

Sectioning

Unfixed sections are cut to determine the pattern of expression of DsRed and cell type specific markers using immunohistochemistry. Embryos are embedded in OCT and frozen in hexane cooled over anacetone-dry ice slurry. Sections are cut at 10 μm using a microm cryostat and collected onto slides.

Immunohistochemistry

Frozen sections or entire embryos are fixed, blocked extensively, followed by primary antibody overnight. The geminin (sc-13015) and BMP4 (sc-6896) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif, USA); the nanog antibody from Kamiya Biomedical Company (Seattle, Wash, USA). Whole mount immunohistochemistry was carried out following [29]. Geminin and nanog primary antibodies were used at 1 : 100, BMP4 at 1 : 50. Secondary antibody-HRP (1 : 200, Jackson Immunoresearch Laboratories,

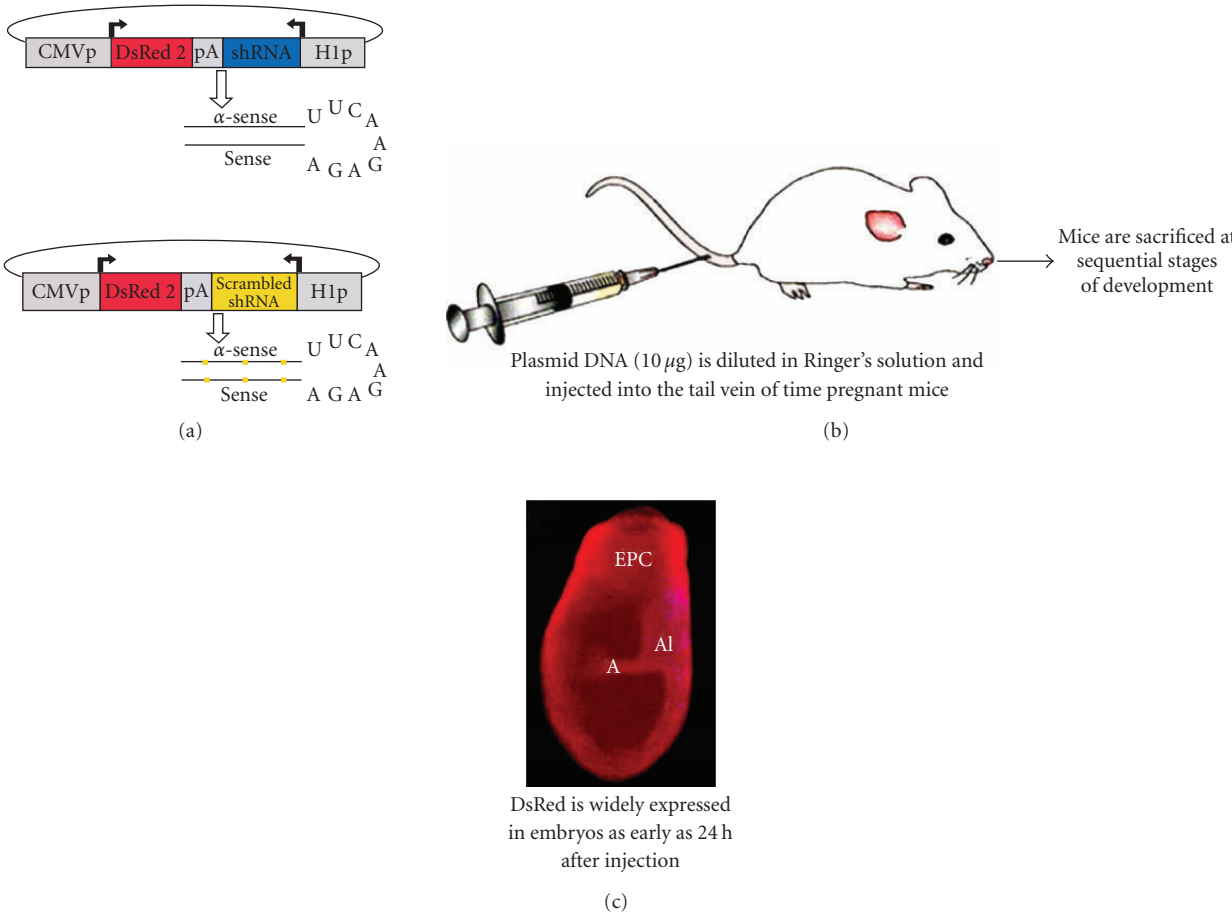

Figure 1: (a) shRNA expression plasmids were constructed using the pCS2 plasmid as the backbone. The DsRed 2.1 coding region was removed from the pDsRed2-1 vector (Clontech) and cloned downstream of the CMV promoter in the MCSI. The mouse H1 promoter (1040–1215 nt) of the RNAseP/PARP2 promoter, GenBank accession AF191547, was PCR-amplified from genomic DNA and cloned into MCSII. Gene-specific shRNAs (blue region) or scrambled shRNAs (yellow) are then ligated downstream of the H1 promoter. (b) Tail vein injections were carried out in pregnant mice as we have done previously (29). (c) Embryos are dissected from the uterus, and decidua and membranes are removed. Transmitted light and fluorescence images of embryos are captured using a Leitz-inverted fluorescence microscope to determine the extent of DsRed expression and to examine their morphology. A: amnion, Al: allantois, EPC: ectoplacental cone.

West Grove, Pa, USA). Images are captured using a Leitz Fluovert or DMIRB microscope then imported into Adobe Photoshop.

PCR

RNAs are extracted from embryos using the Trizol reagent (Invitrogen, Carlsbad, Calif, USA), quantified, and DNAsed. Prior to the reverse transcription (RT) reaction, RNA is subjected to 30 cycles of PCR with β-actin primers to verify that there is no genomic DNA present. RNAs (1 μg) serve as templates in RT reactions with oligo-dT primers. General PCR conditions are 94°/3 m, 94°/1 m, 51–63°/1 m, and 72°/2 m for 25–35 cycles; however, parameters are optimized for each primer pair. The products are electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, then images are scanned into the BioRad Gel Documentation system. For quantitative analysis of gene expression, real-time PCR is performed using the Clonetech Qzyme system on a BioRad iCycler. Real-time PCR primers were designed and optimized by Clontech for use in multiplexed assays with β-actin serving as a reference gene. All reactions are performed in triplicate, and data are analyzed using the $2^{-\Delta\Delta CT}$ method.

RESULTS

We have delivered shRNA to more than 100 pregnant mice, and obtained both gene silencing and expression of the DsRed fluorochrome in embryonic tissues, persisting in postnatal mice. We have carried out a number of experiments to determine if implantation site is correlated with knockdown. In general, embryos implanted near the vagina exhibited greater knockdown than those near the ovaries. In most cases, there is knockdown and DsRed is expressed in embryos. Occasionally ($\sim$ 5% of the injections), there is no

transfection, likely because injection itself fails due to an insufficient amount of DNA entering the circulation.

Geminin shRNA

The geminin gene has been both down regulated and over expressed in Xenopus embryos, reducing or expanding the neural ectoderm fields, respectively [31]. Geminin is particularly interesting because, as suggested by its name, the protein has two functions: the C-terminus functions in cell cycle progression required for differentiation; the N-terminal is involved in early neural differentiation [32]. Despite its provocative expression in the early neural ectoderm and demonstrated role in amphibian, *Drosophila*, and zebrafish development, there is not yet a knockout of *geminin* in the mouse.

When a shRNA targeted to *geminin* was delivered on E6.5, and embryos were examined one–three days later, we observed reductions in neural tissue, neural tube closure defects that typically affected the midbrain, and posterior neuropore. In early embryos, we observed abnormally expanded nodes and failure of closure of the primitive gut endoderm (Figure 2). When we examined geminin expression in whole mount immunohistochemistry, wild-type embryos were indistinguishable from control embryos exposed to the scrambled hairpin both in morphology and in the pattern of geminin protein expression in the newly induced neural ectoderm (Figures 2(a), 2(b)). Geminin was present at slightly higher levels in the anterior neural folds compared with the posterior region of early somite-staged control embryos (Figures 2(a), 2(b)). There was a slight geminin immunoreactivity in the neural ectoderm of some *geminin*-targeted embryos (Figure 2(c)); while others expressed virtually no geminin protein (Figure 2(d)). When semiquantitative RT-PCR was carried out on RNA isolated from individual embryos from three litters, there was some variability in knockdown in the shRNA-exposed embryos, with two embryos expressing levels similar to control, others expressing intermediate, low, or no *geminin* mRNA (Figure 3).

Although *geminin* targeting in amphibian and *Drosophila* embryos has axis patterning and neural tissue consequences, there is no information on the early expression of *geminin* or targeted deletion of the *geminin* gene in the early mouse embryo. Since it is strongly induced by noggin, the observed neural, node, and endoderm abnormalities are likely due to the early expression of *geminin* in these tissues.

Nanog shRNA exposure

The *nanog* gene encodes a varient homeodomain protein originally identified in ES cells, where it is required to maintain pluripotency and inhibit lineage differentiation [33]. Targeted deletion in embryos is lethal before implantation [34], but additional evidence suggested that *nanog* is expressed in germ cells and somatic tissues later in development [35]; however, its role could not be assessed due to the early lethality of null embryos. To determine the role of *nanog* in later stages of development, we have exposed 21 litters of pregnant mice to shRNA-targeted to *nanog* via tail vein injection. We have observed widespread resorption of *nanog*-targeted embryos, and in other litters we have observed abnormalities of gastrulation and neurulation. *Nanog* knockdown embryos are characterized by axis abnormalities which are present in early somite embryos, considerably earlier in development than the turning process is initiated, endoderm overgrowth, and neural tube closure defects, particularly of the midbrain neural folds. Somite segmentation is also often abnormal, and we have observed abnormalities of cell migration through the primitive streak at gastrulation. Figures 4 and 9(b) illustrate some of these malformations.

In whole mount immunohistochemistry, nanog protein expression is significantly reduced, particularly in the primitive streak of embryos exposed to the *nanog* shRNA (37). To correlate phenotype and knockdown, we carried out quantitative PCR on RNA from individual *nanog*- targeted embryos from an entire litter. Silencing ranged from complete in three embryos to 60% of wild-type *nanog* levels in the least severely affected embryo. The presence of phenotypic abnormalities correlates strongly with the degree of knockdown, as illustrated in Figure 4 by the largely normal appearance of the embryo from lane 6, compared with the embryo from lane 7.

Somewhat surprisingly, two nanog-shRNA embryos expressed slightly elevated levels of the *Oas1* gene (Figure 5, lane 15). *Bmp4* expression was robust, however, suggesting that there had not been widespread silencing of nontargeted genes. Although it is widely employed to monitor off-target effects [36], *Oas1* is expressed in muscle, brain, and connective tissue during development [37, 38]. In addition, *Oas1* plays a role in cell cycle progression [39], suggesting a need to monitor additional interferon targets in these studies.

Targeting multiple genes: Bmp4, 7RNA interference

Bmp4 has previously been shown to be required in the gastrulation-staged embryo, where it is important in mesoderm differentiation and organization of the primitive streak [40]. Later *Bmp4* plays a role in determining the boundaries of the neural ectoderm and surface ectoderm [41], with particularly high levels of BMP4 associated with regions of epidermal ectoderm differentiation.

When a cocktail of shRNA targeted to *Bmp4* (exons 2 and 3) was delivered on E6.75 of gestation to pregnant mice, we observed defects of neural tube closure, allantois development, and of heart and axial rotation (Figure 6(b)) in targeted embryos. The number of primordial germ cells identified by alkaline phosphatase staining was also strikingly reduced. RT-PCR analysis of RNA obtained from individual *Bmp4* shRNA-exposed embryos from one entire litter identified only one embryo with any expression of *Bmp4* (Figure 7).

Immunohistochemical localization of BMP4 protein was carried out on sections through shRNA- and pRed (plasmid lacking the hairpin) exposed embryos, and indicated significant depletion of BMP4 in targeted embryos [30]. We also have carried out Western blotting analysis of protein isolated

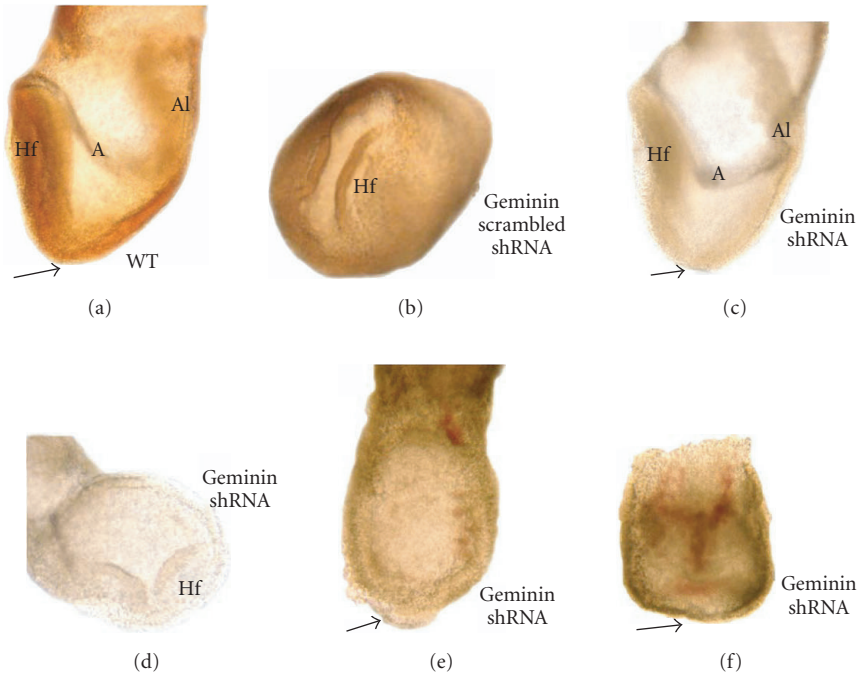

Figure 2: Immunohistochemical localization of geminin in control (a) and (b) embryos, and in embryos exposed on E6.5 to *geminin* shRNA (c and d). In control embryos, both wild type (a) and embryos exposed on E6.5 to a scrambled *geminin* hairpin construct (b), the expression of geminin protein was high in the neural ectoderm of the head folds, although geminin was also expressed in the posterior neural ectoderm as well (brown reaction product). There is a slight background staining of the allantois and membranes in all embryos (a)–(d). (c) and (d) Embryos were exposed to the shRNA-targeting geminin, examined and fixed on E7.5 of gestation, then immunohistochemistry to identify patterns of geminin protein expression was carried out as for (a) and (b) (secondary antibody-HRP). There is low-level geminin protein remaining in the neural ectoderm in embryo (c) less than that in embryo (d). (e) and (f) Transmitted light images of embryos exposed to the *geminin* shRNA on E6.5 and examined on E7.5. (e) Many targeted embryos exhibited axis defects, abnormal expansion of the node (arrow), and in later embryos, the endoderm of the gut tube often failed to close. (f) Occasionally, the embryonic axis appeared very flattened, and there was blood within the amniotic cavity. (a), (c), (e), and (f) are sideviews with anterior located toward the left. (b) is a dorso-lateral view, and (d) is a frontal (coronal) view. A: amnion, Al: allantois, Hf: head folds, WT: wild-type control embryo. Arrows indicate the node.

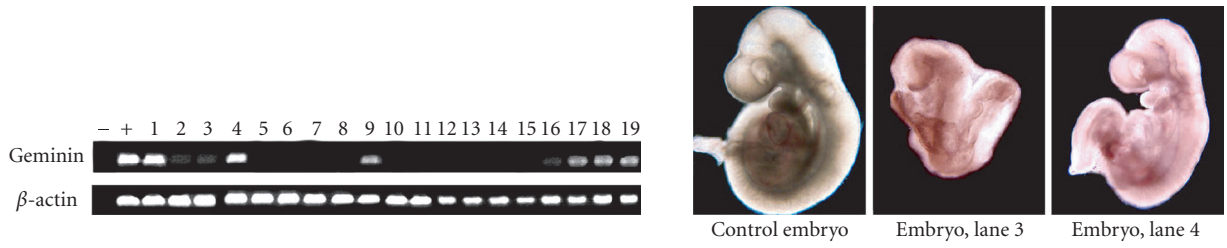

Figure 3: (a) Semiquantitative PCR to detect *geminin* expression in two entire litters of *geminin* shRNA- exposed embryos. Some embryos continue to express nearly normal levels of *geminin* (lanes 1, 4), while others express low (2, 3, 16), intermediate (9, 17–19), or undetectable (5–8, 10–15) levels of *geminin*. The most advanced embryos consistently expressed the highest levels of *geminin*. Two entire litters of *geminin*-targeted embryos were examined; 1–8 and 9–19. – = no RT, + = E9 embryo RNA. (b) Sideview of control and embryos expressing varying levels of *geminin*.

from individual embryos exposed to *Bmp4* shRNA, where there was a reduced expression of phospho-Smads 1/5/8, which are phosphorylated in response to BMP4,7 signaling.

Conventional gene targeting of *Bmp4* results in peri-implantation lethality [40], while on a C57Bl/6 background embryos live until approximately 26 somite stage [41], and are characterized by axis elongation abnormalities. The results of the *Bmp4* RNAi phenocopy many defects in the *Bmp4* null embryos [40, 41] including anomalies of axis formation, primordial germ-cell differentiation, and neural tube closure

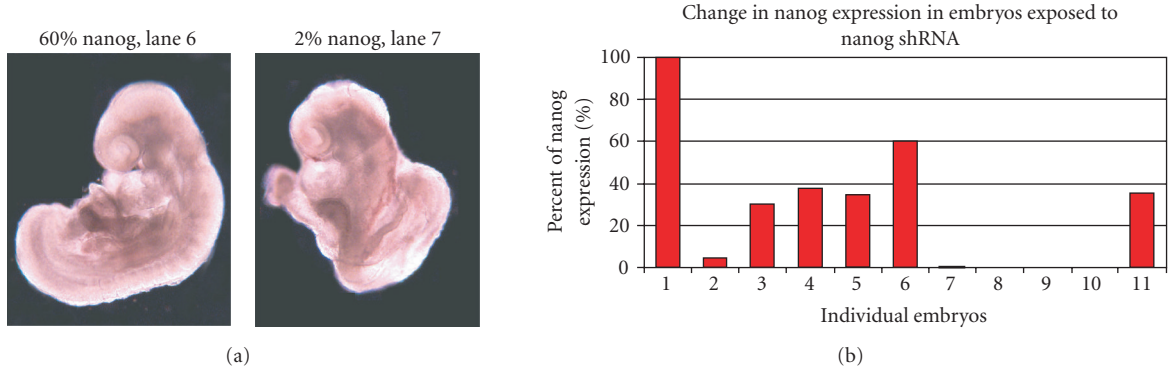

Figure 4: (a) Sideviews of two embryos exposed to *nanog* shRNA. Although the first embryo expressed 60% of wild-type levels of *nanog* mRNA, developmental defects are minor and include an axis abnormality and a flattened posterior neuropore. When *nanog* levels are reduced to 2% of wild type, embryos were more severely affected. The embryo in the right panel is characterized by defects of somite segmentation, neural tube closure, and abnormalities of endoderm differentiation. (b) Q-PCR analysis of *nanog* mRNA expression levels in individual embryos. Embryos were exposed on E6.5 to the *nanog* shRNA and examined on E9.0. cDNA from each embryo was run in triplicate in quantitative PCR with primers to both *nanog* and *β-actin* using the Clontech Qzyme system. Levels of *β-actin* and *nanog* expression from *nanog* shRNA-treated embryos (lanes 2–11) were compared to expression in a control embryo (lane 1). *Nanog* expression ranged from 0–60% of control levels. .

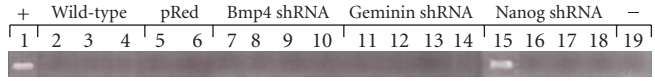

Figure 5: *Oas1* PCR. Single embryo RT reactions were subjected to 40 cycles of PCR with primers for *Oas1* mRNA (71). Mouse brain RT was used as a low-level expression positive control (+). Only 2 *nanog* shRNA embryos were positive for *Oas1* expression (one shown, lane15). – = no cDNA control.

[30]. Many of these are also observed in embryos lacking *Bmpr1a* [42].

Because BMP proteins have overlapping functions in development, we examined the effects of knocking down multiple *Bmps* (Figure 6). We delivered a cocktail of shRNA targeted to *Bmp4* + *Bmp7*, as well as to *Bmp7* alone. The *Bmp7* shRNA embryos were the least severely affected (Figure 6(c)) with neural tube closure defects, while the *Bmp4* + *Bmp7* shRNA embryos had widely expanded neural folds, defects of rotation, failure of development of posterior structures, and ventral body wall closure defects (Figure 6(d)), a more severe phenotype than either the *Bmp4* shRNA or *Bmp7* shRNA embryos, but strikingly similar to the caudal dysgenesis and the "massive brains" reported in *Xenopus* embryos following morpholino depletion of *Bmp2, 4*, and 7 [43].

Durability of the RNAi

To determine how long knockdown could be maintained, we carried out tail vein injection of shRNA targeted to *Bmp4* on E6.5 and examined neonatal mice. On postnatal days 1–5, neonates were characterized by cystic bladders, had rudimentary testes or ovaries, and were consistently growth retarded compared with mice exposed to the pRed control (Figure 8(a)). Expression of DsRed was maintained in many tissues in both the mother (including milk) and in the offspring (Figures 8(b), 8(c), 8(d),8(e), and 8(f)). There were also anomalies of the subventricular neural stem cell zone (SVZ; Figures 8(b), 8(c)) which depends on noggin-BMP4 signaling [44].

Multiple targets, multiple phenotypes

Although there are considerable data available regarding the role of secreted signaling molecules in the initial events of neural induction, very little is known regarding the genes that bridge the process of neural induction and neural differentiation. To identify novel genes that mark the earliest neural ectoderm, we carried out a differential-display RT-PCR screen of genes induced in mouse embryonic stem (ES) cells by noggin protein. From this screen, we selected several transcripts that were expressed in early embryos just after induction. Based on their expression profiles, we selected several candidates for RNAi silencing. Two of these had not previously been examined during development. During the course of our work, *Aggf1* (angiogenic factor with Gpatch and FHA domains 1) was identified as an angiogenic factor mutated in human disease [45], but no information is available about its expression or role in development. Initial in situ hybridization studies indicated that *Aggf1* is expressed at high levels in the distal epiblast, especially in the posterior epiblast on E7.5. At later stages it is expressed in the neural ectoderm. shRNA

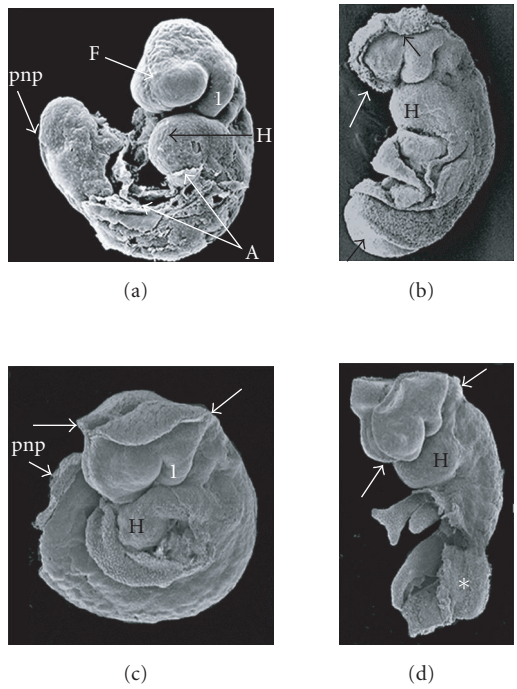

Figure 6: *Effects of Bmp shRNA*. (a) Scanning electron microscopy (SEM) view of a control embryo illustrating completed neural tube closure in the forebrain (F) region; the posterior neuropore (pnp) has not yet been closed. (b) *Bmp4* shRNA-exposed embryo with widely open anterior neural folds (arrows) and posterior neuropore (lower black arrow). (c) SEM view of a *Bmp7* shRNA-exposed embryo. Both the midbrain (arrows) and the posterior neuropore (pnp arrow) are widely open, but the body axis defects characteristic of *Bmp4* shRNA and *Bmp4* + *7* shRNA embryos were not present. (d) Ventrolateral SEM view of a compound *Bmp4* + *Bmp7* shRNA embryo. The cephalic neural folds are unfused (arrows) and the posterior region is rudimentary (∗). 1: first branchial arch, A: amnion, F: forebrain, H: heart, pnp: posterior neuropore.

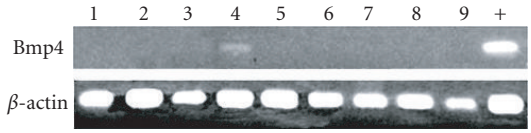

Figure 7: RT-PCR analysis of individual *Bmp4* shRNA-exposed embryos from one entire litter. The positive control (+) is from an embryo exposed to pRed control vector alone. Pregnant dams were injected on E6.5 and RNA extracted from embryos on E9.0.

targeted to Aggf1 produced a lethal phenotype at E7.5. In these embryos, the ectoderm delaminated, and blood was often present within the amniotic cavity. Given its role in vessel formation, it is not surprising that we also observed implantation defects in shRNA-exposed embryos. Rare embryos that survived to E8.5 were characterized by focal hemorrhages and neural tube defects that affected midbrain and posterior neuropore (Figure 9(c)).

Est1 was identified twice in the differential display assay. Initial in situ hybridization localization studies indicate that it is expressed in the early epiblast, preconfiguring the primitive streak, in the node, later in the neural ectoderm. Targeting Est1 produced a severe neurulation phenotype, embryos with open neural folds, defects of embryonic rotation, and differentiation of posterior structures, reminiscent of genes involved in L-R axis patterning (Figure 9(d)).

A number of Wnt family members were also identified in this screen. Because *Wnt8b* had not previously been silenced, we delivered shRNA targeted to *Wnt8b* to pregnant dams on E6.75. Resulting embryos were characterized by axis elongation defects (Figure 9(f)). These embryos also had neural tube closure anomalies and defects in closure of the endoderm.

We have delivered shRNA targeted to *Wnt8b, Bmp4, Bmp7, Bmp4* + *Bmp7, geminin, nanog*, and to two Ests identified in a differential display RT-PCR screen and observed specific targeting and unique phenotypes (Figure 9). These studies have also identified a previously unsuspected role for *nanog* in gastrulation and also in somite organization (Figures 4, 9(b)). Overall, we believe that these results are important and valid for a number of reasons. One, we have targeted multiple genes and observed unique phenotypes. These include *Bmp4* (phenocopies the Bmp4 null embryos, as far as is possible to determine due to early lethality of the null embryos), *Bmp7* alone, *Bmp4* + *Bmp7*, *Wnt8b*, *nanog, Aggf1*, and Est1. Two, in each case where an antibody is available to the protein (BMP4, nanog, geminin) or to the downstream signal transduction cascade (PhosphoSmad1, 5, 8), we have demonstrated knockdown in "individual" embryos. Three, in cases where an antibody is not available, we have demonstrated unique phenotypes and knockdown by PCR. Four, these data also demonstrate that we can knock down multiple targets, for example, *Bmp7* and *Bmp4*, and identify an additive phenotype.

DISCUSSION

With genome-wide gene sequencing data now available, there is increased interest in systematically manipulating "all" the genes of the mouse to understand their roles in development and disease. Many new tools to manipulate gene function have been developed including ribozymes, microRNAs, DNAzymes, as well as a number of methods for post-transcriptional gene silencing such as morpholinos (review [46]), antisense oligos (review [47]), and RNAi (review [3]). RNAi is typically more robust than antisense oligos or morpholinos in embryos [48, 49], and morpholinos have the additional problem that the translational start site must be known, so uncharacterized genes (such as Ests) cannot be targeted.

RNAi may be particularly appropriate in targeting a developmental disease such as Down's syndrome/trisomy 21 once critical duplicated genes are identified, and may also be effective in targeting upstream pathways in metabolic disease to limit disease progression, or in silencing activating gene mutations, such as in the FGF receptor-2 which produces

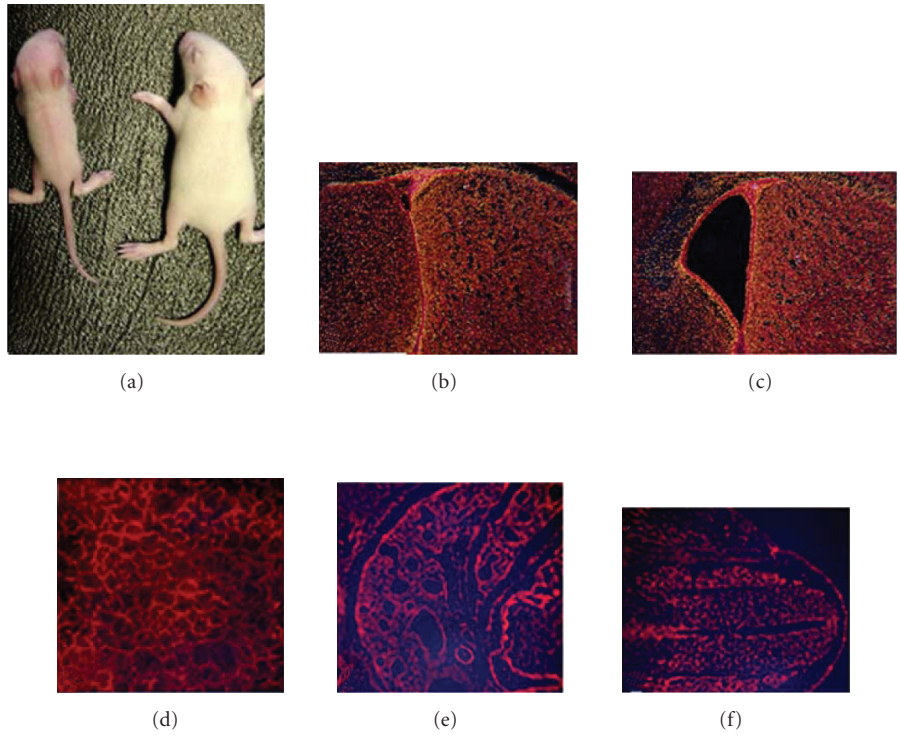

Figure 8: *Longevity of the RNAi.* (a) Day 10 neonatal mice (PN10) obtained from litters exposed on E6.5 to the *Bmp4* shRNA (left) or to pRed (no hairpin plasmid) control (right). *Bmp4* shRNA mice were consistently developmentally delayed and lacked testis or ovaries. (b) and (c) Coronal sections through the lateral ventricles of PN3 mice exposed on E17.5 to pRed control (b) or to *Bmp4* shRNA (c). In addition to the obvious anomalies of the subventricular zone and ventricle, neural stem cells obtained from the *Bmp4* shRNA mice fail to differentiate normally. (d)–(f) illustrate the persistent expression of DsRed in liver (d), lung (e), and spinal cord (f) in neonates exposed to *Bmp4* shRNA on E6.5.

craniosynostosis [50]. Systemic delivery will also be applicable to diseases that affect tissues with open circulations, as well as diseases in which the blood brain barrier is opened such as Duschenne muscular dystrophy, certain brain tumors, in aging, and in multiple sclerosis (review [51]).

These studies have identified unsuspected roles in development for several genes. In the case of *nanog*, which in null embryos is lethal at early cleavage stages of development, we have identified a role in gastrulation, neurulation, and in endoderm differentiation. There is not a report of a knockout of the *geminin* gene in the mouse, and it will be of particular interest to study carefully the characteristics of the neural tissue in targeted embryos, as well as the characteristics of the node. Neither is there a published report of a *Wnt8b* knockout, but many of the defects observed in this study are similar to those present in other Wnt null embryos. For example, *Wnt3a* null embryos have similar severe posterior truncations [52]. The use of RNAi directed against individual Wnt mRNAs should allow rapid analysis of specific Wnt functions. In addition, since Wnts may compensate for each other, masking functions in single-gene knockouts, combinatorial Wnt RNAi should help elucidate overlapping relationships between the Wnts. Delivery of shRNA to pregnant dams has also provided an opportunity to rapidly determine if there was a role in early embryos for novel genes identified in a differential display RT-PCR screen. A role for *Aggf1* in later aspects of vasculogenesis was described previously [45], and given its role in vessel development, it is not surprising that targeting *Aggf1* affected the implantation process.

The ability to target multiple genes with overlapping expression and function, as in the case of *Bmp4/7* [53], is an important improvement over traditional knockouts in which mutations in multiple genes are obtained by breeding. In the future, it will be important to target multiple genes using a single plasmid containing multiple hairpins, rather than the cocktail we have employed to target *Bmp4* and *Bmp7*.

To date, study of the placental transport of plasmid DNA has come largely from attempts to deliver pDNA for in utero gene therapy, which have produced conflicting results. Thus, when pDNA complexed with liposomes was delivered by intravenous injection of pregnant mice on E2.5, 5.5, 8.5, 11.5, or 14.5, no plasmid DNA was detected in fetuses exposed on E2.5 or E5.5, while embryonic expression peaked with delivery on E8.5, compared with E11.5 or E14.5. "All" embryos treated on E8.5 expressed the plasmid, with sustained expression at 40 days postinjection [54]. However, it has also been reported that DNA-liposome complexes were trapped in the visceral endoderm prior to placenta development on E11.5 [55]. Others have also reported hemodynamic transfer of genes to the fetal compartment, however. For example,

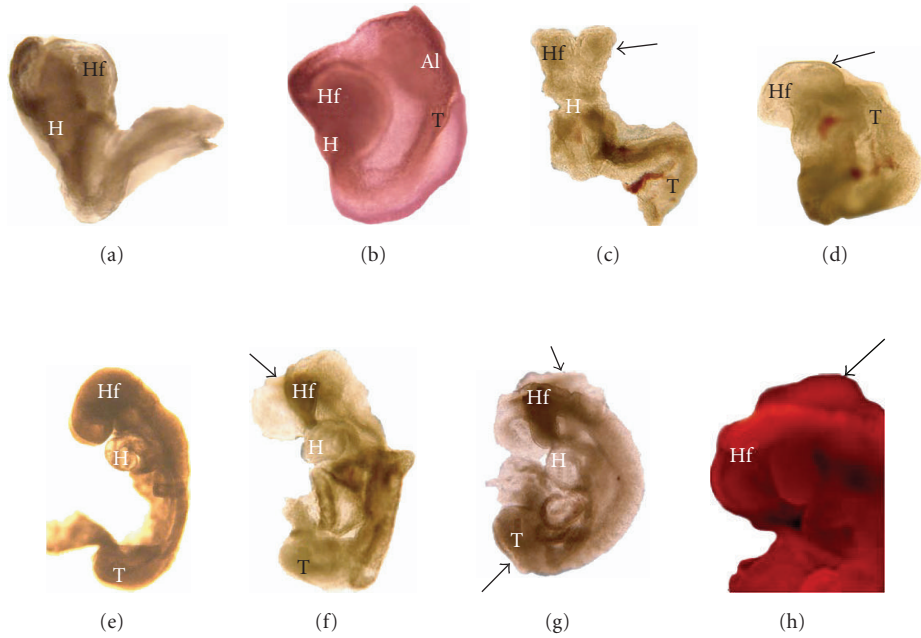

Figure 9: *Control- and Gene-targeted embryos:* ***nanog, geminin, Aggf1*, and Est1.** (a) Control embryo. Sideview of an embryo exposed to pRed plasmid (no hairpin) on E6.5 and examined on E8.5, illustrating the normal appearance of the head folds (Hf), somites, and unturned body axis. (b) *Nanog* shRNA. Embryo exposed on E6.5 to shRNA targeting *nanog*, illustrating the typical lack of development of the head folds (Hf) and posterior region in the tail bud (T). Somites have also failed to segregate normally. (c) and (d) We carried out a differential display RT-PCR screen of genes induced in D3 ESC by noggin exposure, then targeted two using tail vein injection of shRNAs. (c) *Aggf1*-targeted embryos failed to implant normally and the primitive ectoderm often delaminated into the amniotic cavity. Hemorrhages are present within the embryo; there are striking abnormalities of turning and posterior development in the rare embryo that survived to E8.5. (d) Est1-targeted embryo. There were anomalies of primitive streak organization in these embryos. They also often failed to turn to adopt the fetal position and exhibited abnormalities of the node. (e) pRed control. Sideview of an E8.5 pRed (no hairpin) control. This embryo is beginning the turning process, the body axis is elongated, neural folds are fused in the anterior (head fold, Hf) region, although the posterior neuropore remains open in the tail bud (T). (f) *Wnt8b*-targeted embryo illustrating the shortened axis and open neural folds typical of these embryos. (g) and (h) *Geminin* shRNA. An embryo exposed to *geminin* shRNA on E6.5 and examined on E8.5. There are very characteristic midbrain (upper arrows) and posterior neuropore (lower arrow in (g)) defects in these embryos, which exhibit widespread DsRed fluorescence (H). All embryos are oriented with anterior toward the left. Al: allantois, H: heart, Hf: head folds, T: tail bud. Arrows indicate open neural folds.

intravenous delivery of plasmid DNA to pregnant mice on E9.5 successfully immunized the fetuses against HIV-1 and influenza [56]. We have avoided carriers since liposomes are often immunogenic, are generally less effective in serum, and can be toxic to both the embryo and the pregnant female [55].

Although we have obtained widespread expression of our construct, a number of improvements and alternative approaches can be considered. It would be possible to increase the amount of DNA injected, although 5 μg plasmid DNA was optimal (saturating) and > 25 μg/mouse was toxic [57, 58]. Other studies have shown that transfection efficiency is not determined by volume or rate, but the amount of DNA delivered, with highest expression achieved with 1000 ng/mouse (23). Given the ~ 1.6 mL blood volume of an 18 g mouse and observations that there is less degradation of pDNA in a larger volume of carrier [58], increasing the volume delivered would be an option. Rate of injection—5 seconds is better than 30 [24, 58]—could also be considered, but very rapid injection can be lethal.

Despite careful breeding, the developmental stage of individual embryos at the time of exposure to shRNAs cannot be known precisely, and may account for some of the variability in our results. Alternatives include using exo-utero surgery of midgestation embryos with injection of shRNAs and electroporation [49]. For early postimplantation stages when exo-utero surgery is not applicable, whole embryo culture presents another option [59]. Better promoters and better control of CRE expression in the early embryonic compartment will allow the development of hybrid approaches to specifically, inducibly silence gene expression in a particular tissue/cell type (eg, 61). Interestingly, the oocyte-restricted ZP3 promoter was recently employed to drive expression of dsRNA targeted to the *Mos* gene, recapitulating the null phenotype, with spontaneous parthenogenetic activation [60]. These and other recent investigations suggest that it will be possible to target RNAi to particular cells or tissues.

One drawback to tail vein injection is the loss of plasmid DNA to the female and unintended transfection of maternal

Table 1

Aggf1	tttacttcgaccatgacttgca gctcgtcctttctatcgaggc	tttaTttcgCccatgacttgTa gcGcgtcctGtctatcgagTc
Bmp4	cagtccatgattcttggga ccttcagaaaccggtcggc	cagtcAatgaGtcttgTga cAttcagaaTccggtAggc
Bmp7	tccatctccgtagtatccg ttcgacgacagctctaatg	tccGtctccgCagtatTcg ttcCacgaTagctctCatg
Est1	cccgaacgatatttggtgtga caagacgacgtgacaattcca	ccGgaacgaGatttggtgtTa caCgacgaTgtgacaattcTa
Geminin	tcatgtacacggcctagcat attcctgactatccggtga	tAatgtacCcggcctagAat atCcctgactatAcggcga
Nanog	ttctgggaacgcctcatcaatgc ggaagcagaagatgcggactgt	ttcCgggaacAcctcatcaTt ggaTgcagaCgatgTggactgt
Wnt8b	atgtacaccctgactagaaactgcagcct gtccgctgcgagcagtgccgccgg	aGgtacaccAtgactagatgcagAct gGccgctAcgagTagtgccgccgA

PCR Primers

Gene	Forward 5′-3′	Reverse 5′-3′
Bmp4	ctcccaagaatcatggactg	aaagcagagctctcactggt
Geminin	gagaaaatgagttgccaaaagg	ccacagcttgaagtctgagatg
Nanog	agggtctgctactgagatgctctg	atcttctgcttcctggcaag
Oas1g	atggtagtatcaataagaagc	gcatagacagtgagtagctcc
actin	ttgctgacaggatgcagaaggaga	actcctgcttgctgatccacatct

tissues. Since the liver has an expandable circulation and is easily transfected using intravenous delivery, it is important to monitor liver function in pregnant females and neonates. Obviously, when the targeted gene is important in maternal tissues, this is a larger concern that must be constantly monitored. Additional experiments might therefore include targeting of a nonessential protein such as EGFP in the GFPU mouse [61] which has no known downstream targets, nor have there been deleterious effects of EGFP cleavage products. It would be possible to mate hemizygous GFPU mice to determine if there are any deleterious effects that are transmitted to the nontargeted +/+ embryos. It would also be useful to target a gene expressed only in male embryos, so that female littermates would serve as a control for off-target and/or maternal effects.

It is impractical to carry out microarray analyses of individual, targeted embryos to determine specificity of targeting, although in previous studies when the targeting construct was specific, RNAi signatures were unique and highly specific for the target gene [62, 63]. More detailed analysis can also be carried out to verify the presence of specific mRNA cleavage products using 5′ RACE, PCR to identify the cleavage fragments with sequencing [64]. It has generally been assumed that early development in the embryo is incapable of mounting a full interferon response [65], yet interferon responsive genes such as fragilis are expressed during very early postimplantation development [66]. Since *Oas1* may have additional roles in development, monitoring other interferon-responsive genes would also be appropriate in these studies. Recent evidence also suggests that shRNA expression can competitively inhibit endogenous miRNA function via exportin 5 [67], although inclusion of scrambled hairpin constructs should control for this effect. Much remains to be understood about this technique, particularly regarding transport, uptake, and expression in the embryos and fetuses.

Since the first transgenic mouse was developed in 1980 by pronuclear injection of DNA [68], there have been major improvements to the technological base for mouse functional genomics, and RNAi promises to be a powerful new addition to that tool set.

ACKNOWLEDGMENTS

This work is supported by NIH Grants NS-048187, NS-39438. The authors are grateful to JM Velkey and MH Morell for discussions and technical advice, to David Turner for the pCS2 plasmid, and to A George for unpublished data on primordial germ cell numbers.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 28945, Pages 1–9
DOI 10.1155/JBB/2006/28945

Research Article

Adenovirus-Mediated In Vivo Silencing of Anaphylatoxin Receptor C5aR

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Received 20 January 2006; Accepted 5 April 2006

C5a, one of the most potent inflammatory peptides, induces its inflammatory functions by interacting with C5a receptor (C5aR) that belongs to the rhodopsin family of seven-transmembrane G protein-coupled receptors. C5a/C5aR signaling has been implicated in the pathogenesis of many inflammatory and immunological diseases such as sepsis and acute lung injury. Widespread upregulation of C5aR has been seen at both the protein level and transcriptional level under pathological conditions. Here, we show that C5aR gene expression can be specifically suppressed by siRNA, both in vitro and in vivo. A panel of chemically siRNA oligonucleotides was first synthesized to identify the functional siRNA sequences. The short hairpin RNAs (shRNAs) were also designed, cloned, and tested for the silencing effects in C5aR transfected cells. The effective shRNA expression cassettes were then transferred to an adenovirus DNA vector. ShRNA-expressing adenoviruses were intratracheally administered into mouse lung, and a significant in vivo silencing of C5aR was obtained four days after administration. Thus, C5aR shRNA-expressing adenoviruses appear to be an alternative strategy for the treatment of complement-induced disorders.

INTRODUCTION

The complement system was initially identified as an important innate immune mechanism of host defense to eradicate microbial pathogens. Recently, complement activation has been implicated in the pathogenesis of many inflammatory and immunological diseases, including sepsis [1], acute respiratory distress syndrome [2], rheumatoid arthritis [3], glomerulonephritis [4], multiple sclerosis [5], ischemia-reperfusion injury [6], and asthma [7]. Complement activation exerts its harmful roles through the generation of complement protein split products, especially C3a and C5a (also known as anaphylatoxins). C5a induces its inflammatory functions by interacting with C5aR that belongs to the rhodopsin family of seven-transmembrane G protein-coupled receptors [8–10]. Traditionally, C5aR expression was thought to be present only on hemopoietic cells, bone marrow cells [11], neutrophils [12], monocytes [13], and eosinophils [14]. However, recent studies have demonstrated the presence of C5aR on nonmyeloid cells, including cells in human lung and liver [15–17], rodent type II alveolar epithelial cells [18], astrocytes [19], kidney tubular epithelial cells [20], mesangial cells [21], and hepatocyte-derived cell lines [22, 23]. Widespread upregulation of C5aR has been seen in organs (heart, liver, lungs, kidneys) from septic animals [24].

Due to the detrimental effects of complement activation under pathologic conditions, interventions aimed at blocking C5a/C5aR signaling represent promising targets for therapeutic treatment in the inflammatory disorders. Peptide antagonist (C5aRa) to the C5aR markedly reduced the lung permeability index (extravascular leakage of albumin) in mice after intrapulmonary deposition of IgG immune complexes [25]. C5aRa treatment substantially reduced I/R-induced pathological markers [26, 27]. In addition, mice injected at the start of CLP with a blocking antibody to C5aR showed dramatically improved survival [24].

RNA interference (RNAi) is an emerging technology that specifically inhibits target gene expression in vitro and in vivo. Tuschl and colleagues demonstrated that exogenously introduced short (19–23 nt) synthetic RNA oligonucleotides can silence genes in somatic cells without activating nonspecific suppression by dsRNA-dependent protein kinases [28]. Successful gene silencing has been achieved in vivo by intravenous injection of siRNA oligos in a large volume of saline solution [29–31] or by injecting smaller volumes of siRNAs that are packaged in cationic liposomes [32]. However, these strategies are limited by the in vivo stability of siRNA molecules and the efficiency with which they are taken up by target cells and tissues. DNA vector-based siRNA expression system would facilitate transfection experiments in

cell cultures, and allow the use of transgenic or viral delivery systems [33–36]. Several viral vectors have been used to induce RNAi silencing in cultured cells and in experimental animals, including lentivirus [37, 38], retrovirus [33], adenovirus [39, 40] and adeovirus-associated viruses (AAV) [41, 42]. Adenoviruses can infect a wide range of cells and have been shown to silence gene expression in vivo [39, 43, 44]. In this study, we demonstrated that systemic application of an adenovirus expressed siRNA can specifically inhibit C5aR gene expression in vivo.

MATERIALS AND METHODS

Cells and antibodies

Mouse alveolar macrophages (MHS cell line) were purchased from ATCC and was cultured in RPMI1640 medium (Life Technologies) supplemented with 10% fetal calf serum as well as 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml), and 0.05 mM 2-mercaptoethanol. HEK293 cell was cultured in DMEM medium (Life Technologies) supplemented with 10% fetal calf serum.

Anti-mouse C5aR polyclonal antibody was generated against a 37 aa peptide spanning the N terminus of the mouse C5aR (MDPIDNSSFEINYDHYGTMDPNIPADGIHLPKRQPGDC) [45]. The antipeptide specific Ab was purified by affinity chromatography using the synthetic peptide coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ). HA antibody (12CA5) was obtained from BABCO (Berkeley Antibody Company).

Cecal ligation puncture-induced sepsis

C57BL/6 male mice (6 to 8 wk of age weighing 25–30 g; Jackson Laboratories, Bar Harbor, ME) were used in all experiments. Mice were anesthetized with ketamine. A 1 cm long midline incision was made to expose the cecum and adjoin the intestine. With a 4–0 silk suture, the cecum was tightly ligated below the ileocecal valve without causing bowel obstruction. The cecum was punctured through with a 21 gauge needle and gently squeezed to extrude luminal contents, ensuring patency of the two puncture holes. The abdominal incision was then closed with a 4–0 nylon suture and skin metallic clips (Ethicon, Somerville, NY). Sham-operated animals underwent the same procedure except for ligation and puncture of the cecum.

Cloning of mouse C5aR

According to the mouse C5aR sequence [46], two primers (forward primer: 5′-CGG AAT TCC GAT GGA CCC CAT AGA TAA CAG C-3′; reverse primer: 5′-GAA GAT CTT CTA CAC CGC CTG ACT CTT CCG-3′) were designed to amplify mouse C5aR from mouse liver RNA using reverse transcription-polymerase chain reaction. PCR products were digested with *Eco*R I and Bgl II and then cloned into pCMV-HA, a mammalian expression vector that contains the hemagglutinin epitope (PYDVPDYA).

Table 1: Sequences and locations of siRNA oligos.

No.	Sense sequences (5′ − > 3′)	Locations
1	CGCCAUCUGGUUUCUGAAUd(TT)	210
2	CUACUGGUACUUUGAUGCCd(TT)	297
3	ACAUCUGCUACACCUUCCUd(TT)	656
4	CCCUAUCAUCUACGUCAUGd(TT)	888

siRNA oligos

The 21 nt sense and antisense siRNA oligomers targeting against mouse C5aR mRNA were designed and synthesized by Qiagen. Their locations and sequences are shown in Table 1 (only the sense sequences are shown). The oligos were numbered based on the nucleotide position within the coding region of mouse C5aR sequence. Sense and antisense oligos were annealed in HEPES buffer (100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) to obtain siRNA duplexes. Rhodamine labeled control (nonsilencing) siRNA was also purchased from Qiagen.

Cell transfection and western blot

For MHS cell transfection, cells were plated in 6-well plates (8×10^5/well) and transfected with 6 μl of TransIT-TKO (Mirus) and 30 pmol of siRNA duplexes. Silencing effects were detected by semiquantitative RT-PCR two days after transfection. For HEK293 cell transfection, cells plated in 35 mm dishes (5×10^5 cells/dish) were transfected with HA-tagged C5aR using Lipofectamine 2000(Invitrogen). Two days after transfection, cells were placed in lysis buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 2 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, and 1 mM PMSF. Thirty microliters of the whole cell lysates were electrophoresed in 10% SDS-PAGE and then transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with TBST (40 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.1% Tween 20), containing 5% nonfat dry milk for 1 hour at room temperature. The membrane was then incubated with anti-mouse C5aR serum (1:500 dilution) overnight at 4°C. After three washes in TBST, the membrane was then incubated in a 1:10 000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia). The membrane was developed by enhance chemiluminescence according to the protocol of the manufacturer (Amersham Pharmacia).

Detection of C5aR mRNA by semiquantitative RT-PCR

Total RNA was isolated from cells or lung tissue with the Trizol reagent according to the manufacturer's instructions (Invitrogen). Digestion of any contaminating DNA was achieved by treatment of samples with RQI RNase-free DNase (Promega). RT-PCR was performed with 1 μg of total RNA using the one-step RT-PCR system (Invitrogen) according to the protocol of the manufacturer. Primers for

Table 2: Sequences and locations of short hairpin RNAs (note: "**G**" indicates an extra nucleotide added to the target sequence).

No.	Hairpin sequences (5′− > 3′)	Locations
1	gatcc**G**CTACTGGTACTTTGATGC*tt*caagaga[a](AS[b])TTTTTg	300
2	gatccGCCCATCTGGTGTCAGAAG*tt*caagaga[a](AS[b])TTTTTg	420
3	gatccGTGTACCGGGAGGCATAA*tt*caagaga[a](AS[b])TTTTTg	517
4	gatcc**G**ACATCTGCTACACCTTCCT*tt*caagaga[a](AS[b])TTTTTg	656
5	gatccGAGGGTGGAGAAGCTGAAC*tt*caagaga[a](AS[b])TTTTTg	831
6	gatcc**G**CCCTATCATCTACGTCATG*tt*caagaga[a](AS[b])TTTTTg	888

[a] hairpin loop sequence.

[b] antisense sequence.

C5aR were

(i) forward primer: 5′-GTTGCAGCCCTTATCATCTA-C-3′,
(ii) reverse primer: 5′-TTCCGGGTTGAGGTGTCGTCT-G-3′.

The primers were designed for a 908 bp DNA fragment amplification (nucleotides 112-1019). The primers for the "housekeeping" gene GAPDH were

(i) forward primer: 5′-ACCACCATGGAGAAGGCTGC-3′,
(ii) reverse primer: 5′-CTCAGTGTAGCCCAGGATGC-3′.

After a reverse transcription step for 30 min at 50°C, 25–35 cycles were used for amplification with a melting temperature of 94°C, an annealing temperature of 60°C, and an extending temperature of 72°C, each for 30 seconds, followed by a final extension at 72°C for 7 min. RT-PCR products were confirmed by electrophoresis of samples in 1% agarose gel. To ensure that DNA was detected at the linear part of the amplification curves, PCR was performed with different cycle numbers for C5aR and GAPDH primers. Thirty cycles were used for C5aR amplification in CLP mice, and thirty-two cycles were used in control mice. Twenty five cycles for GAPDH were found to be in the linear range of PCR amplifications.

Immunocytochemistry and confocal microscopy

HEK293 cells were plated on glass bottom 6-well plates (no. 1 thickness coverslips). Two days after transfection, cells were fixed in paraformaldehyde. Fluorescence microscopy was performed as previously described [47]. HA-tagged C5aR was visualized with the affinity purified anti-mouse C5aR antibody (1:500 dilutions) and goat anti-rabbit Alexa 568 (Molecular Probe) secondary antibody (1:1000 dilutions) in the lissamine-rhodamine channel. Cells were imaged on a LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) with a 63 × water lens.

Plasmids expressing short hairpin RNAs

Vectors that express C5aR short hairpin RNAs (shRNAs) under the control of U6 promoter were constructed by inserting pairs of annealed DNA oligonucleotides into the linearized RNAi-Ready pSIREN-DNR-DsRed-Express Vector (BD knockout adenoviral system 2) between the BamH I and EcoR I sites. Sequences and locations of shRNAs are shown in Table 2 (only the top strands are shown).

Generation of siRNA-expressing adenoviruses

U6-driven shRNA cassettes and the CMV-driven DsRed expression cassette in pSIREN-DNR-DsRed donor vector ware transferred to the adenoviral acceptor vector pLP-Adeno-X-PRLS by cre-loxP mediated recombination according to the protocol of the manufacturer. HEK293 cells were transfected with Pac I-digested adenoviral DNA using lipofectamine 2000. One week after transfection, cytopathic effect (CPE) was detected and cells were spun down and lysed in 500 μl PBS with three consecutive freeze-thaw cycles. Supernatants containing infectious adenoviruses were amplified twice by infecting larger scale of HEK293 cells. Viruses were purified by column (Puresyn, Inc) and concentrated by YM-50 centricon (Millipore). Titers of the viruses were determined by Adeno-X rapid titer kit (BD clontech).

Isolation of peritoneal macrophages and adenovirus infection

Macrophages were isolated from the peritoneal cavities of 4- to 6-week-old C57BL/6 mice 4 days after intraperitoneal injection of 0.5 ml 3% thioglycollate, yielding ≥ 95% macrophages as demonstrated by cytospin and differential stain analysis. The cells were seeded at a density of 2×10^6 cells/ml and plated into 6-well plates at 2 ml/well [48] in the same culture medium as MHS cells.

MHS cells and peritoneal macrophages plated in 6-well plates were infected with 100- to 2000-MOI of adenoviruses in a volume of 150 μl of culture medium for one-hour. During the one hour incubation, plates were shaked occasionally at a 15 min interval. Cells were changed to 2 ml fresh medium after the incubation and cultured for another two days for the examination of silencing effects.

Adenovirus-mediated siRNA delivery in animals

Eight- to 10-week-old C57BL/6 mice (weighing 25–30 g) were used in this study. The 50 μl viral suspensions with a

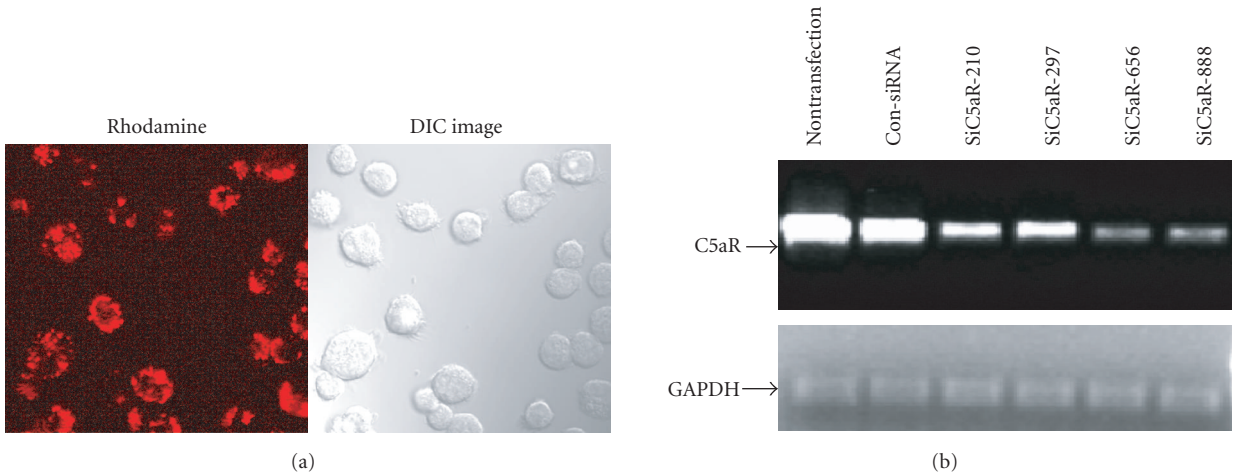

FIGURE 1: Screening of functional mouse C5aR siRNA oligos in MHS cells. MHS cells in 6-well plates were transfected with 30 pmol of control siRNA and four C5aR siRNA oligos using Mirus TransIT-TKO transfection reagent. (a) Eight hours after transfection, cells transfected with Rhodamine-labeled control siRNA were plated on glass-bottom plate and washed twice with phenol-red-free medium and subjected to confocal microscopy analysis (Zeiss). The transfected cells were visualized by red fluorescence (left). (b) Total RNA extracted from transfected MHS cells was quantified for C5aR mRNA expression by semiquantitative one-step RT-PCR (upper panel). GAPDH was used as endogenous control (lower panel).

dosage of 1×10^9 plaque-forming units (pfu) were injected intracheally into mouse lungs. Four days after the injection, mouse lung were extensively flushed with DPBS, and frozen in liquid nitrogen. The 2 ml Trizol reagent was added into one lung for RNA isolation procedure.

RESULTS AND DISCUSSION

siRNA duplexes efficiently inhibited endogenous C5aR in MHS cells

The 21- to 23-nucleotide siRNAs were generated by ribonuclease III through cleavage of longer dsRNAs. They have been shown to act as the mediators of post-transcriptional gene silencing in cells and animals [49, 50]. For the initial screening of the functional siRNA sequences of mouse C5aR, we used synthesized 21-nucleotide siRNA duplexes with 3′-(dTT) overhangs (Table 1) to transiently transfect MHS cell, a cell line that expresses C5aR mRNA endogenously.

Similar to other macrophages, none of the standard transfection methods (e.g., calcium phosphate, lipid, or electroporation) can efficiently transfer DNA plasmids into MHS cells. As a minimum, seventy percentage transfection efficiency is required to study the silencing effects. We used rhodamine-labeled control siRNA to determine the transfection efficiency. Different from larger DNA plasmids, short siRNA oligos could be efficiently transferred into MHS cells by a lipid-mediated method (TransIT-TKO). All of the cells showed red fluorescence eight hours after transfection (Figure 1(a)). No fluorescence was observed for the control cells without TransIT-TKO reagent (data not shown). To confirm that these oligos actually entered the cells, a Z-stack protocol of confocal microscopy was performed and the scanning results confirmed the cytosolic localization of the fluorescence-labeled siRNA.

All four synthesized siRNA duplexes showed silencing effects on the endogenous C5aR expression as examined by semiquantitative RT-PCR (Figure 1(b)). SiC5aR-210 and siC5aR-297 had moderate inhibition effects, whereas siC5aR-656 and siC5aR-888 suppressed 90 percentage of the endogenous gene. No silencing effects were observed for the control siRNA (scrambled sequences). The specificity of these siRNA oligos was verified by BLAST search against the gene bank.

Cotransfection of siRNA duplexes inhibits C5aR protein expression in HEK293 cells

C5aR is a member of the seven transmembrane receptor superfamily and is ubiquitously expressed on neutrophils, macrophages, thymocytes, epithelial, and endothelial cells. However, in vitro cultured cell lines have very low or non-detectable expression of the receptor. To determine if these siRNA duplexes could also suppress C5aR protein expression, full-length mouse C5aR cDNA was cloned into a HA-tagged mammalian expression vector and transfected into HEK293 cells. Immunocytochemistry analysis showed that this C5aR construct showed a cortical pattern of expression on the membranes of HEK293 cells (Figure 2(a)). Western-blot analysis using anti-mouse C5aR antibody revealed a $\sim$ 45 kDa band, which is consistent to the size of the receptor expressed in tissues and primary cells (Figure 2(b)) [51].

In the cotransfection experiment, 0.8 μg C5aR plasmid was transfected with 40 pmol of control siRNA or the C5aR-siRNAs. Two days after transfection, these cell lysates were analyzed by Western blot. Compared to control group, both siRNAs (siC5aR-656 and siC5aR-888) could significantly

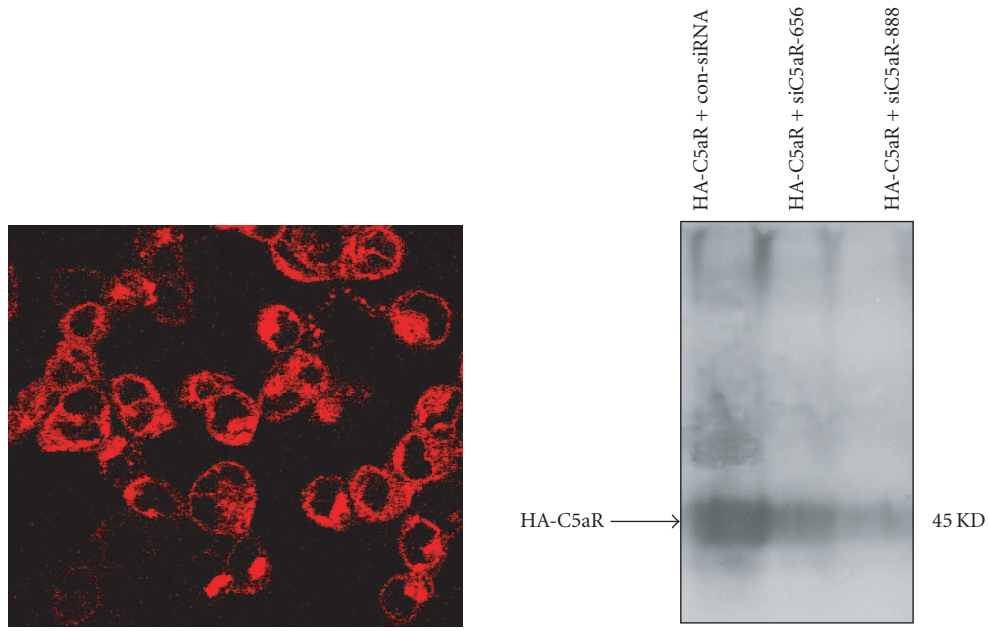

Figure 2: Oligo siRNA inhibition of C5aR protein expression in HEK293 cells. HEK293 cells were cotransfected with HA-tagged C5aR plasmid, control siRNA, or C5aR siRNA oligos. (a) Forty-eight hours after transfection, transfected cells were fixed in paraformaldehyde and stained with the anti-mouse C5aR primary antibody and goat anti-rabbit Alexa 568 secondary antibody. Expression and localization of C5aR were visualized by confocal microscope under lissamine-rhodamine channel (Zeiss). (b) Cell lysates from transfected cells were subjected to Western-blot analysis using anti-mouse C5aR antibody (1:500).

inhibit the protein expression of C5aR in HEK293 cells (Figure 2(b)).

Hairpin RNA constructs inhibit C5aR expression

Based on the identified C5aR siRNA oligonucleotide sequences, we designed short hairpin RNAs (shC5aR-656 and shC5aR-888) according to the design rules suggested by the manufacturer (BD PharMingen) and inserted them into a U6 promoter-driven shRNA expression donor vector, pSiren-DNR-dsRed. For the most efficient transcription initiation of RNA polymerase III, an extra "**G**" was added at the 5′ end of the sense sequence (Table 2). Sense- and antisense nucleotides were separated by a 9 nt spacer and five consecutive Ts were added at the 3′ end for the termination of short RNA transcripts.

To evaluate the silencing effects of these short hairpins, a 1:10 (HA-C5aR to hairpin RNA) ratio of plasmids was used for the cotransfection experiment in HEK293 cells. A luciferase short hairpin construct pSiren-shLuc served as negative control. Unexpectedly, neither one of the C5aR hairpin constructs (pSiren-shC5aR-888 and pSiren-shC5aR-656) efficiently inhibited C5aR expression (Figure 3).

Different from synthesized siRNA oligos, the effects of DNA vector-based hairpin RNAs are regulated by multiple components. Target sequence selection is an important component, while other factors such as the transcription efficiency, the cleavage efficiency of hairpin RNA into siRNA by Dicer [33], and the subcellular localization of the short transcript [52], can also affect the efficacy of a hairpin RNA. To select an effective hairpin RNA structure that could be used for our in vivo adenoviral delivery, four additional plasmids, pSiren-shC5aR-300, pSiren-shC5aR-420, pSiren-shC-5aR-517 and pSiren-shC5aR-831 were constructed. pSiren-shC5aR-831 (third bar) and pSiren-shC5aR-517 (fifth bar) strongly inhibited C5aR expression in HEK293 cells, while pSiren-shC5aR-420 (sixth bar) and pSiren-shC5aR-300 (seventh bar) had little inhibitory effects (Figure 3). No extra "**G**" was added to pSiren-shC5aR-420, -517 and -831 as the target sequence itself start with a "G". An extra "**G**" was added to pSiren-shC5aR-300 (Table 2).

In vitro and in vivo silencing effects of adenovirus-expressed siRNA

pSiren-DNR-DsRed is an intermediate vector of adenoviral DNA. After we identified two functional shRNAs (C5aR-517 and C5aR-831), the U6 promoter and the hairpin cassette in the donor vector were transferred to a promoterless adenoviral acceptor vector by cre-loxp mediated recombination. The adenoviral DNAs were then transfected into HEK293 cells to produce infectious viruses. Two adenoviruses (adeno-shC5aR-517 and adeno-shC5aR-831) and one control virus (adeno-shLuc) were generated for in vivo gene silencing.

Macrophages that express C5aR endogenously were used to test the silencing effects of these viruses. However, these

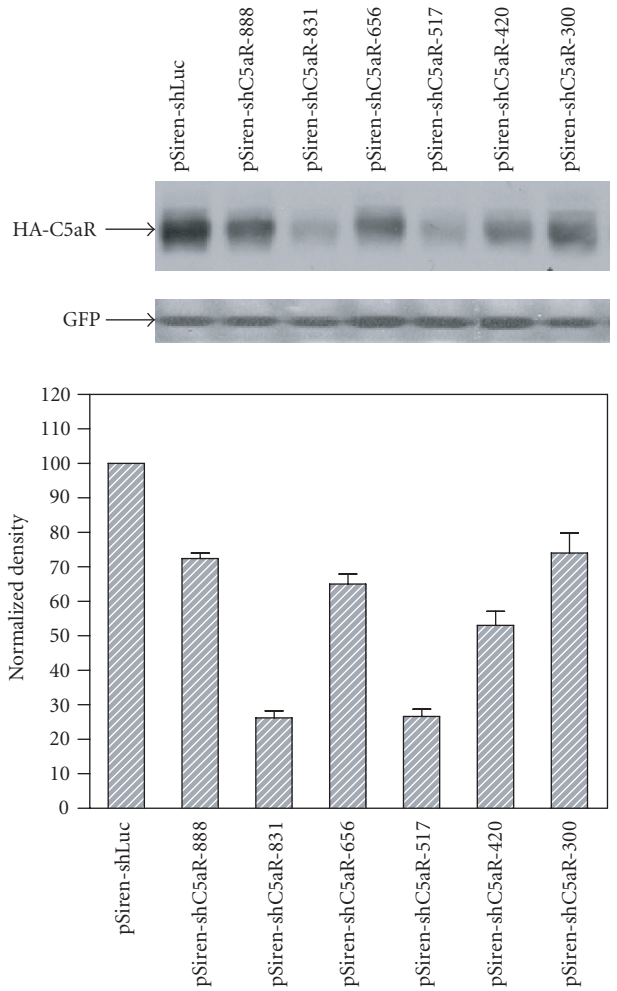

Figure 3: Silencing effects of plasmid-derived short hairpin RNAs. HEK293 cells in 6-well plates were transfected with 4 μg of a 10:1 mix of pSIREN-DNR-DsRed-shRNA to HA-tagged C5aR together with 0.3 μg gfp plasmid. Forty-eight hours after transfection, cell lysates were analyzed by Western-bloting for HA (upper panel). Expression of gfp showed equal transfection efficiency. The bottom panel showed the quantitative densitometric analysis of bands shown in the upper panel. The percentage of inhibition was normalized to control cells transfected with luciferase-shRNA. Averages are derived from 3 to 5 independent experiments (standard deviations are indicated).

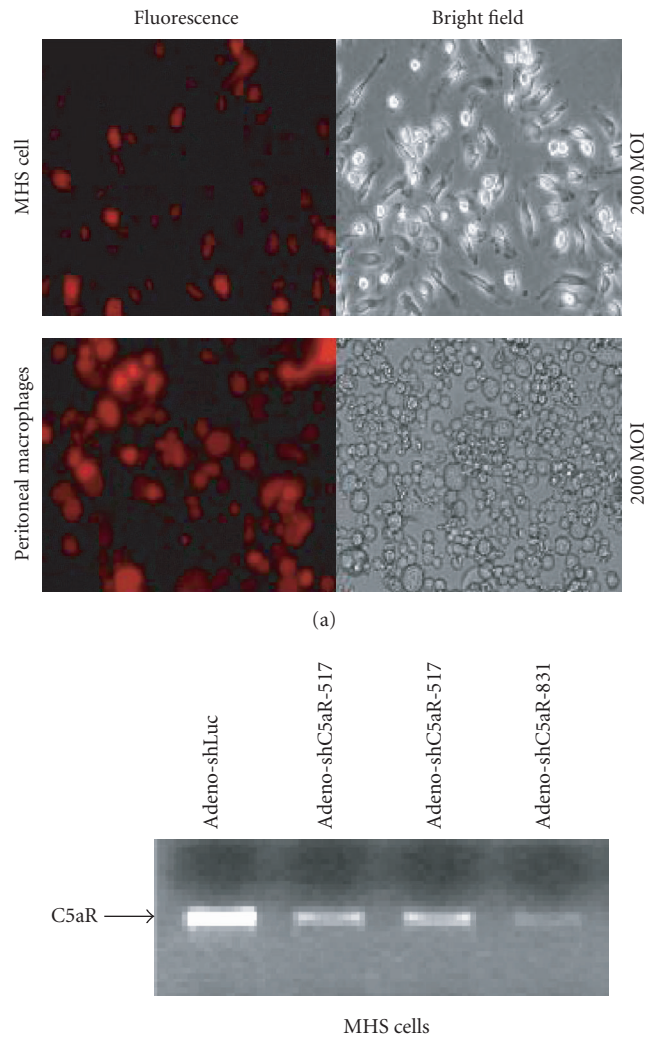

Figure 4: In vitro silencing by siRNA-expressing adenovirus. Both MHS cell and peritoneal macrophages were infected with 2000 MOI of adenovirus. (a) Sixty hours after infection, expression of dsRed protein in the cells was visualized by fluorescence microscope. Virus infection efficiency was examined by comparing the fluorescence images (left) and the bright field images (right). (b) Silencing effects of adenovirus-expressed siRNAs were examined by one-step RT-PCR as described in Material and Method. Adeno-luciferase siRNA was used as control.

cells do not express coxsackie receptor [53, 54] and they internalize adenovirus about 100- to 1000-fold less than receptor-expressing cells, such as epithelial cells [55]. To identify an optimal infection condition, we tested a range of infectious units (100, 500, 1000, and 2000 MOI) and found that $\sim$ 80% of the cells could be infected (as shown by the DsRed marker in the adenoviral DNA) at 2000 MOI (Figure 4), whereas less than half the cells were infected at 1000 MOI (data not shown). In addition to the high infectious units, we also used a low volume of medium during the incubation to enhance other virion uptake pathways (endocytosis or phagocytosis). C5aR mRNA expression in infected MHS cells were examined by semiquantitative RT-PCR. Both adeno-shC5aR-517 and adeno-shC5aR-831 effectively inhibited endogenously expressed C5aR and the inhibition effect of adeno-shC5aR-831 appeared to be stronger (Figure 4(b)).

To evaluate the ability of virally expressed siRNAs to diminish target gene in vivo, mice were injected intratracheally with 1×10^9 plaque-forming unit (pfu) infectious units of recombinant adenovirus expressing shC5aR-831 or the control virus adeno-shLuc. Four days after infection, RNAs were isolated from mouse lungs and subjected to RT-PCR analysis. As shown in Figure 5(b), the luciferase control virus infection did not change C5aR expression in the lung tissue. However,

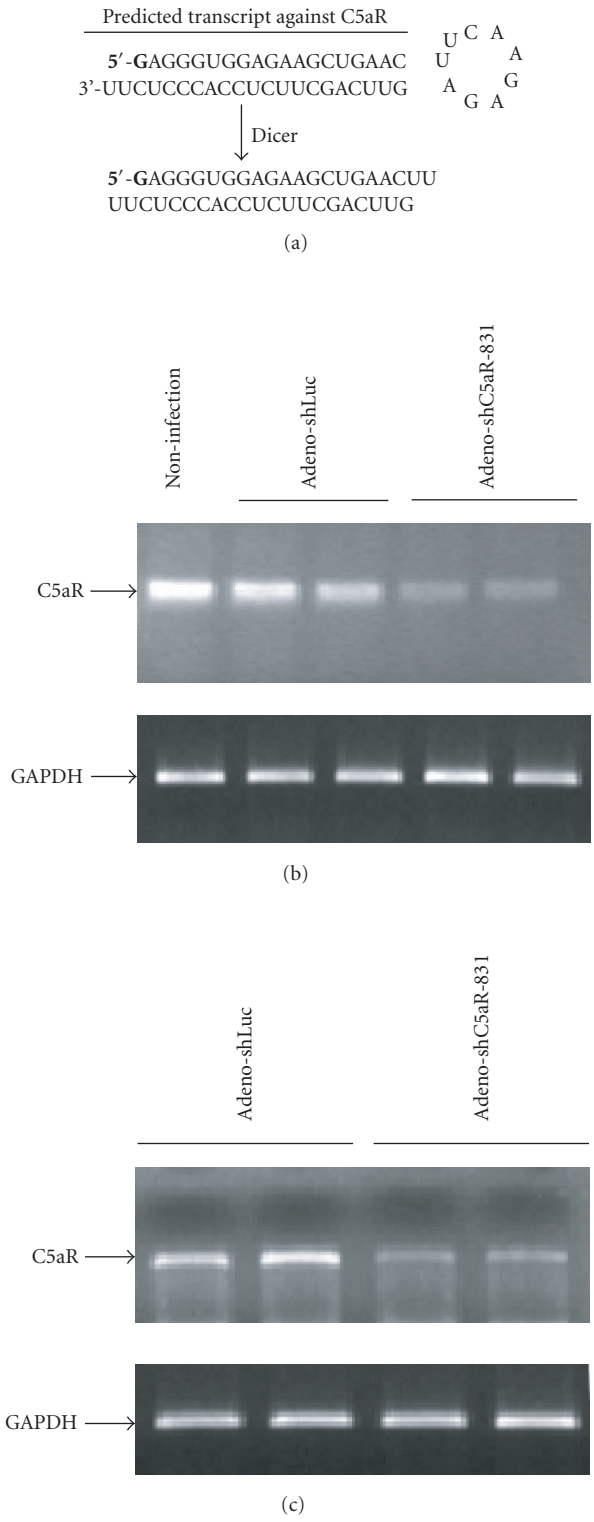

Figure 5: In vivo silencing by adenovirus delivey of siRNA. (a) The predicted shRNA transcript from the adenovirus vector and the expected Dicer processing products in vivo. Semiquantitative RT-PCR analysis with whole lung RNAs from control (b) and septic mice (c) showed the decreased C5aR expression in mice infected with C5aR-siRNA adenovirus. Luciferase-siRNA-adenovirus infected lungs were used as controls.

adeno-shC5aR-831effectively inhibited C5aR expression in the lung tissue. To test the effect of adeno-shC5aR-831 in disease condition, sepsis was induced by CLP in mice that had received adenovirus for four days. Twenty four hours after CLP, RNAs from lungs were analyzed for C5aR and GAPDH expression. As shown in Figure 5(c), the inhibitory effect of adeno-shC5aR-831 is still effective under sepsis condition. These data indicate that complement receptor C5aR could be suppressed in vivo by an adenovirus-mediated siRNA knockdown strategy under both normal and disease conditions.

RNA interference is a powerful tool to silence gene expression post-transcriptionally. Different from gene knock out, the inhibition efficiency of siRNAs could vary dramatically by employing a different delivery method and sequence design strategy of siRNA oligonucleotides or short hairpins. In this study, it is noted that the vector-based siRNA sequences could not be simply derived from chemically synthesized oligo sequences. It may be due to the fact that the functionality of shRNAs depends on more complicated intracellular mechanisms. In fact, none of the current design rules guarantee an effective siRNA and a functional siRNA can only be identified experimentally. Another important factor that affects the application of siRNA is the efficiency and the effectiveness of delivery routes. Here we demonstrated the intratracheal administration of siRNA-expressing adenovirus that could efficiently knock down C5aR expression. Thus, C5aR siRNA-expressing adenovirus shall not only serve as a useful tool for studying the mechanisms of complement activation in inflammation, but may also have important therapeutic applications.

ACKNOWLEDGMENT

This work is supported by the National Institutes of Health (Grants GM-61656 and HL-31963).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 69616, Pages 1–20
DOI 10.1155/JBB/2006/69616

Review Article

MicroRNAs in Gene Regulation: When the Smallest Governs It All

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Received 30 January 2006; Accepted 17 April 2006

Encoded by the genome of most eukaryotes examined so far, microRNAs (miRNAs) are small ~21-nucleotide (nt) noncoding RNAs (ncRNAs) derived from a biosynthetic cascade involving sequential processing steps executed by the ribonucleases (RNases) III Drosha and Dicer. Following their recent identification, miRNAs have rapidly taken the center stage as key regulators of gene expression. In this review, we will summarize our current knowledge of the miRNA biosynthetic pathway and its protein components, as well as the processes it regulates via miRNAs, which are known to exert a variety of biological functions in eukaryotes. Although the relative importance of miRNAs remains to be fully appreciated, deregulated protein expression resulting from either dysfunctional miRNA biogenesis or abnormal miRNA-based gene regulation may represent a key etiologic factor in several, as yet unidentified, diseases. Hence is our need to better understand the complexity of the basic mechanisms underlying miRNA biogenesis and function.

INTRODUCTION

In 1990, a group of plant biologists attempted to accentuate the purple pigmentation of petunia petals by increasing the synthesis of anthocyanin via overexpression of a transgene encoding chalcone synthase. Unexpectedly, this transgene induced the formation of white flowers, in association with a block in pigment synthesis and a 50-fold reduction in transgene mRNA levels; this intriguing phenomenon was termed cosuppression [1, 2].

Three years later, in the field of developmental biology, Lee et al [3] identified two lin-4 transcripts, with the smaller (~21 nt) being complementary to seven repeated sequences in the 3′ nontranslated region (NTR) of the heterochronic gene lin-14 mRNA, identified previously by Wightman et al [4]. These findings suggested that lin-4 could regulate lin-14 translation via an antisense RNA:mRNA interaction and play an important role in developmental timing in the nematode *Caenorhabditis elegans* (C elegans) [3, 5].

These studies converged in 1998, when Fire et al [6] obtained evidences about the involvement of double-stranded (ds) RNA intermediates in a phenomenon termed RNA interference (RNAi). The authors noticed that dsRNA species induced a more potent genetic interference than either strands alone in *C elegans*. A year later, while investigating posttranscriptional gene silencing (PTGS) as a natural antiviral defense mechanism, Hamilton and Baulcombe [7] observed the presence of antisense viral RNA of ~25 nt in virus-infected plants. The authors noted that these small RNAs were long enough to convey sequence specificity and suggested that they may be important specificity determinants of PTGS. Subsequent papers reporting that dsRNA-induced mRNA degradation is mediated by 21 to 23 nt RNAs [8, 9] prompted molecular biologists and geneticists to search for the endogenous source of small RNAs. In 2001, three independent groups defined miRNAs as a novel family of small (~22 nt) endogenous RNAs that are diverse in sequence and expression patterns, evolutionarily widespread, and involved in sequence-specific, posttranscriptional regulatory mechanisms of gene expression [10–12].

We now know that miRNA genes are encoded in the genome of most eukaryotic organisms and transcribed by RNA polymerase (pol) II into primary miRNAs (pri-miRNAs). These structured RNAs are then processed by the nuclear RNase III Drosha, acting in concert with the DiGeorge syndrome critical region 8 (DGCR8) protein within a

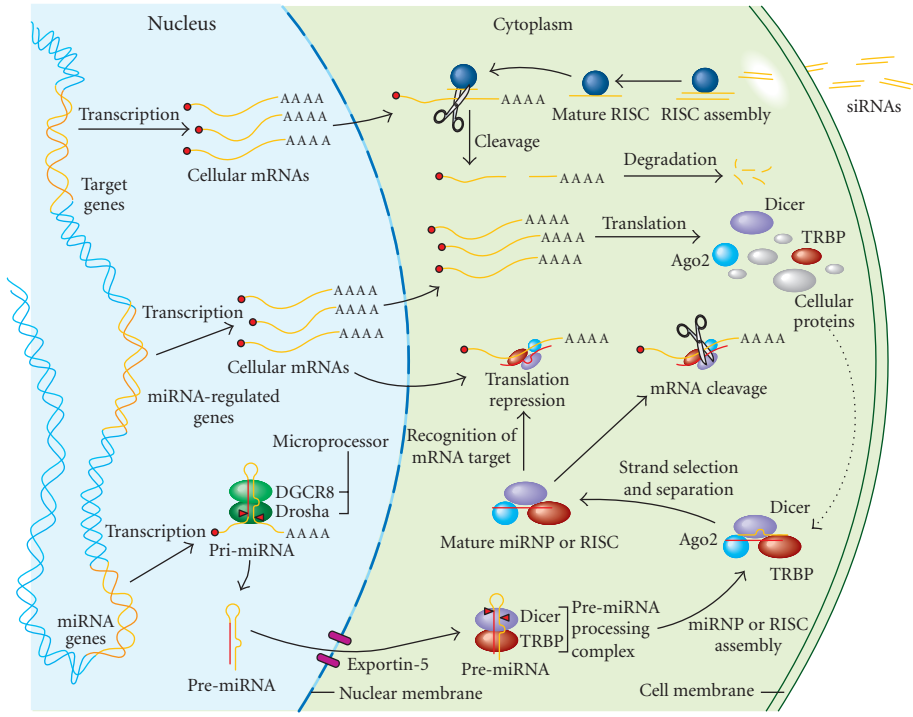

Figure 1: mRNA regulation mediated by microRNAs (miRNAs). miRNA genes are transcribed by RNA polymerase II into primary miRNAs (pri-miRNAs), which are processed by Drosha, acting in concert with DiGeorge syndrome critical region 8 (DGCR8) protein within the microprocessor complex, into ~60 to 70 nt miRNA precursors (pre-miRNAs). Following export via exportin-5, pre-miRNAs are cleaved by Dicer, acting in concert with transactivating response RNA-binding protein (TRBP) within the pre-miRNA processing complex, to generate an imperfect miRNA:miRNA* duplex of ~21 to 23 nt. After a strand selection/separation process, the mature miRNA is loaded into an effector miRNA-containing ribonucleoprotein (miRNP) complex that will recognize and mediate repression or cleavage of specific mRNAs. Synthetic small interfering RNAs (siRNAs) can be introduced into cells and be incorporated into the endogenous miRNA-guided RNA silencing machinery to mediate cleavage of the targeted mRNA.

complex known as the microprocessor [13–16], into ~60 to 70 nt stem loop miRNA precursors (pre-miRNAs) [17–19]. Following export from the nucleus to the cytoplasm by the Ran-GTP dependent transporter Exportin-5 [20–23], the pre-miRNAs are cleaved at the base of the loop by a second RNase III enzyme located in the cytoplasm, Dicer, to generate an imperfect miRNA:miRNA* duplex of ~21 to 24 nt. Dicer was recently shown to act together with the transactivating response RNA-binding protein (TRBP) within a pre-miRNA processing complex. Following strand selection/separation, mature ~22 nt miRNAs are incorporated into, and guide, effector miRNA-containing ribonucleoprotein (miRNP) complexes containing Argonaute 2 (Ago2) towards specific mRNAs. Dicer and TRBP have recently been shown to be a part of a functional human RNA-induced silencing complex (RISC), thereby coupling the initiation and effector steps of RNAi [24]. The targeted mRNA will be initially subjected either to cleavage or translation repression, depending on whether the miRNA:mRNA pairing is perfect or not [25]. The miRNA-guided RNA silencing pathway is illustrated in Figure 1.

In humans, conservative predictions indicate that up to 30% of the genes may be regulated by such a mechanism! Thus, potentially all the cellular pathways may be governed by miRNAs, which may contribute to the fine tuning of gene expression on a global level. The importance of miRNAs in gene regulation will be better appreciated when their function or deregulation, or that of the cellular machinery mediating their biosynthesis and function, will be identified among the underlying causes of several genetic disorders. Indeed, it is easy to conceive that protein overexpression resulting from defective miRNA-based mRNA regulation may compromise normal cell function and cause genetic diseases. In turn, the responsible gene(s) may be responsive to RNAi-based inactivation, illustrating the transition from fundamental research to clinical applications of RNAi.

Today, the miRNA mimetics small interfering RNAs (siRNAs) are increasingly important molecular tools, as they often are the method of choice used by researchers that aim at elucidating the function of a gene. More importantly, perhaps, is the high potential of the approach for therapeutic applications, leading several biotechnology firms to develop and refine tools, and improve the design of new therapeutic strategies in order to take advantage of the natural RNA silencing machinery to silence the expression of disease-causing genes. This requires a better understanding of the miRNA-based RNA silencing machinery in human.

COMPONENTS OF THE RNAi MACHINERY

The endogenous miRNA-guided RNA silencing machinery is composed of several different proteins, protein complexes, and types of RNAs. How these elements integrate with each other to form this important functional cascade is the subject of intense investigations. We will first discuss the protein components, identified so far, that are governing miRNA biogenesis and function (see Figure 1). The subsequent sections will cover the identification of miRNAs and their targets, the biological roles of miRNAs, as well as their involvement in diseases.

RNA polymerase II

RNA pol II, which governs the transcription of protein-encoding messenger RNAs (mRNAs), has been identified as the major transcriptional unit for miRNA genes [17, 26] after some speculations about the potential implication of RNA pol III [25]. The pri-miRNA transcripts, which can be longer than 1000 nts [26] and up to several kilobases long, possess the signature of RNA pol II characterized by a 5′ 7-methyl guanylate (m7G) cap and a 3′ poly(A) tail [17]. Although miRNA genes can be found as clusters forming their own transcriptional units [19, 26], ∼ 40% are transcribed from the intronic sequence of protein-encoding genes [27, 28]. A study by Rodriguez et al [27] has shown that the expression of a large subset of mammalian miRNAs may be transcriptionally linked to the expression of other genes, coding for both proteins and ncRNAs. Although the majority of pri-miRNAs are noncoding RNAs, whose genomic regions do not correlate with known transcripts [29], some of them contain open reading frame (ORF) susceptible to be translated. However, analysis of both endogenous and overexpressed pri-miRNAs showed that very little full-length pri-miRNA transcripts reached the cytoplasm, probably because they were processed by Drosha before they could be exported from the nucleus [17].

Drosha

The RNase III Drosha is a class II endoribonuclease that was identified, cloned, and first implicated in preribosomal RNA (pre-rRNA) processing [30] (see Figure 2). Members of the class II RNase III family are characterized by a duplication of the RNase III domain (RIIID), a C-terminal dsRNA binding domain (dsRBD), as well as a proline-rich region (PRR) and an arginine/serine(RS)-rich domain in the N-terminal region [18, 30]. Previously known as the human RNase III, Drosha was further identified as the enzyme mediating the first step in miRNA biogenesis through conversion of pri-miRNAs into pre-miRNAs [18] (see Table 1), confirming previous findings obtained with nuclear fractions of human cultured cells [19].

Drosha homologues are expressed in *C elegans* [13, 31], *Drosophila melanogaster* (*D melanogaster*) [13, 31], and *Mus musculus* [32], but not in *Schizosaccharomyces pombe* (*S pombe*) (http://www.sanger.ac.uk/Projects/S_pombe/) and *Arabidopsis thaliana* (*A thaliana*) [30]. The absence of Drosha in lower species reveals fundamental differences in the initiation steps of small regulatory RNA biosynthesis, which may have been evolved during the course of evolution.

Pri-miRNA processing by Drosha yields a pre-miRNA product with termini bearing the signature of RNases III, that is, a 5′ phosphate and 2 nt overhangs at the 3′ hydroxylated end [18, 19, 33]. From the junction of the loop and the adjacent stem, Drosha cleaves pri-miRNAs after approximately two helical turns into the stem to produce ∼70 nt pre-miRNAs [18]. Lee et al used deletional mutagenesis on miR-30a followed by in vitro processing to show that sequences covering ∼20 nt upstream and ∼25 nt downstream of the expected cleavage site were necessary and sufficient to support processing [18]. Beyond the pre-miRNA cleavage sites, approximately one helix turn of stem extension is also essential for efficient processing. While Drosha cleavage sites are determined largely by the distance from the terminal loop, variations in stem structure and sequence around the cleavage site can fine-tune the actual cleavage sites chosen [34]. A cleaving model was proposed in which the two RIIID of Drosha form an intramolecular dimer to create a catalytic site for substrate processing [15]. This model is analogous to that proposed for Dicer [35]. The two RIIIDs of human Drosha are distinct in their roles within the dimer: the RIIIDa cuts the 3′ strand, while the RIIIDb cleaves the 5′ strand, independently of each other [15]. Han et al suggested that Drosha may reorientate itself after the recognition of the 3′ end of pri-miRNAs [15] and, as for human Dicer, places the processing center at < 20 base pairs from the terminus [35].

Fractionation of HEK 293 cell nuclear extracts by gel filtration chromatography identified a pri-miRNA processing activity corresponding to a molecular mass of >700 kDa [15]. This activity peak shifted to <650 kDa following treatment of the extract with RNase A, indicating that Drosha may function in a large complex of < 650 kDa. Analysis of Drosha immunoprecipitates by mass spectrometry revealed the presence of DGCR8 in that complex [13, 14]. A distinct, larger Drosha complex containing the DEAD box RNA helicase DDX17/P72, the heterogeneous nuclear ribonucleoprotein M4 (hnRNPM4), and the protein product of Ewing's sarcoma gene (EWS) was reported [14]. As reviewed in Arvand and Denny [36], EWS belongs to a family of genes that encode proteins that may serve as adapters between the RNA pol II complex and RNA splicing factors. Because Drosha has also been previously shown to participate in pre-rRNA processing [30], this large Drosha complex has been suggested to mediate such pre-rRNA processing activities [14].

DGCR8/microprocessor

DGCR8 was identified in anti-Flag immunoprecipitates prepared from an HEK-293 cell line stably expressing Flag-Drosha [14]. This Drosha-DGCR8 complex, which has also been observed in other organisms [13, 16], has been termed microprocessor [14]. DGCR8 contains two dsRBDs and a WW domain that could interact with the N-terminal proline-rich region of Drosha [14].

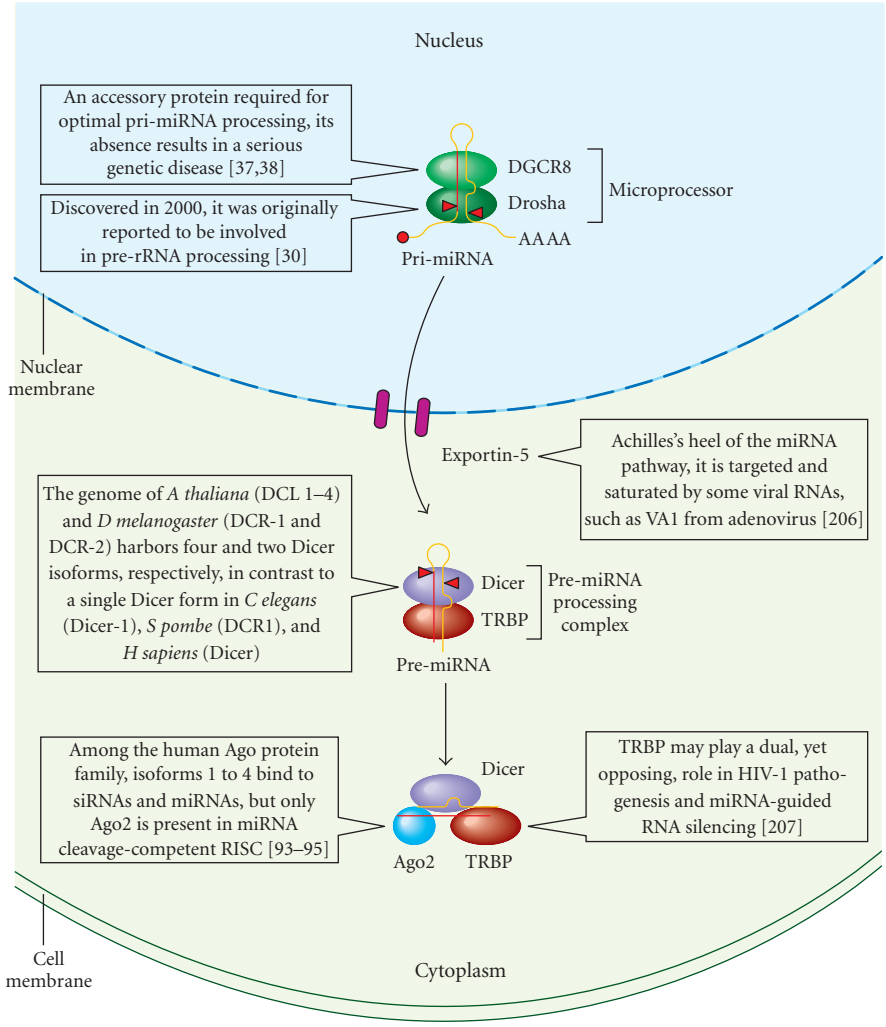

Figure 2: Some peculiarities of the major protein components of the microRNA-guided RNA silencing.

The exact role of DGCR8 in the microprocessor complex remains uncertain, but may be related to pri-miRNA recognition. In accordance with a proposed model, DGCR8 would interact with the stem, and perhaps also the single-stranded (ss) region of this structure, to guide the recognition of the pri-miRNA stem by Drosha dsRBD. Alternatively, but not exclusively, DGCR8 may also interact with the ss region of RNA to correctly orient the complex on pri-miRNAs [15]. Gregory et al [14] have shown that the knock-down of DGCR8 results in, as observed upon Drosha depletion, a pronounced decrease in mature miRNA level. Depletion of both Drosha and DGCR8 resulted in a substantial accumulation of pri-miRNAs, showing the requirement of the microprocessor complex for miRNA processing in vivo [14].

The DGCR8 gene is located in the q11.2 region of the human chromosome 22 that contains ~30 genes and is a part of a common monoallelic deletion [37]. Patients carrying this heterozygous deletion and other chromosomal abnormalities in this region display clinical phenotypes defined as the DiGeorge syndrome, Conotroncal anomaly face syndrome, and Velocardiofacial syndrome [38]. Congenital heart defects, characteristic facial appearance, immunodeficiency, and behavioral problems are other manifestations of these genetic disorders [38].

Exportin-5

Subcellular localization studies previously showed that pri-miRNA and pre-miRNA processing is compartmentalized into the nucleus and cytoplasm, respectively [19], suggesting the existence of a pre-miRNA nuclear export step. Less than two years later, three independent groups reported the identification of Exportin-5, a member of the nuclear karyopherin β transporter family [21], as the nuclear pre-miRNA transporter [20, 22, 23].

Exportin-5-mediated pre-miRNA transport was either reduced upon downregulation of Exportin-5 by siRNAs [23] or enhanced upon overexpression in mammalian cells [39]. Export of pre-miRNAs was greatly reduced by the inhibition of the Ran guanine nucleotide exchange factor, suggesting

Table 1: Characteristics of the major protein components of the miRNA-guided RNA silencing pathway.

Protein	Role/ function	Accession number (mRNA)	mRNA (bp)	Accession number (protein)	Amino acids	Molecular weight (kDa)*	Isoelectric point*	Intracellular localization
Drosha	Processing of pri-miRNA into pre-miRNA	NM_013235	4764	NP_037367	1374	159	7.81	Nuclear
DGCR8	Assistance of Drosha function in the microprocessor	NM_022720	4461	NP_073557	773	86	5.94	Nuclear
Exportin-5	Nuclear export of pre-miRNA	NM_020750	5231	NP_065801	1204	136	5.79	Nuclear membrane
Dicer	Processing of pre-miRNA into miRNA:miRNA*	NM_177438	10 276	NP_803187	1922	219	5.68	Cytoplasmic, ER
TRBP	Assistance of Dicer function in pre-miRNA processing complex	M60801	1368	AAA36765	345	38	7.38	Cytoplasmic
Ago2	Component of miRNPs, repression of translation	NM_012154	3567	NP_036286	859	97	9.19	Cytoplasmic
FMRP	Component of miRNPs, repression of translation	NM_002024	4362	NP_002015	632	71	7.42	Cytoplasmic, nuclear

*Calculated from protein calculator v.3.2 (http://www.scripps.edu/~cdputnam/protcalc.html). ER, endoplasmic reticulum.

that it is catalyzed by RanGTP [22]. In fact, RanGTP was necessary for specific binding of pre-miRNAs by Exportin-5 [23]. Recognition of pre-miRNAs bearing a 2 nt 3′ overhang by Exportin-5 was superior than pre-miRNAs with 5′ or no overhangs [40]. As for the pre-miRNA terminal loop and stem, which should be more than 16 nt in length, their recognition is not sequence-specific [20, 40]. Recognition of a minihelix motif in the RNA allows transport by Exportin-5, as demonstrated by the efficient transport of VA1 RNA from adenovirus 5 [41]. The exact coordination links between Exportin-5 and the nuclear and cytoplasmic steps of miRNA biogenesis remain obscure and need further investigation.

Dicer

Dicer is a ribonuclease III that was first identified as an enzyme capable of generating ~21–23 nt RNA guide sequences from dsRNA to initiate RNAi in *Drosophila* S2 cells [42]. Within a two-month period, three papers reported that null mutations in the Dicer gene altered developmental timing, in association with defective miRNA maturation and accumulation of pre-miRNAs, in *C elegans* [43, 44] and *Drosophila* [45]. Human Dicer cDNA, which had been identified two years before [46], was later cloned and the recombinant protein expressed, allowing the characterization of its RNA binding properties and RNase activity [47, 48]. Localized mainly in the cytoplasm [49] or the endoplasmic reticulum [47] of cultured cells, human Dicer is a large protein composed of several domains: an N-terminal putative ATPase/helicase domain containing a DECH box, a domain of unknown function (DUF283), a PIWI/Ago/Zwille (PAZ) domain, and a C-terminal RIIID, composed of tandem RNase III motifs and a C-terminal dsRBD [35, 42, 47, 48].

Recently, data reported by Zhang et al [35] pointed towards the existence of a single catalytic center in human Dicer. The authors proposed a model in which Dicer would function through intramolecular dimerization of its two RIIID, assisted by the flanking RNA binding domains, PAZ, and dsRBD. The PAZ domain of Dicer may participate in the recognition of the terminal 3′ overhangs of its pre-miRNA substrate [35]. In this model, each RIIID cuts a single strand of the RNA duplex substrate after two turns of α-helices, at the end opposite to that cleaved by Drosha, to produce a new end bearing a hydroxylated 2 nt 3′ overhang and a phosphorylated 5′ end. The 2 nt overhang is measured by the alignment of the dimer rather than by the distance between active residues on one peptide chain, whereas the length of the product (~21 nt) is determined by the distance between the PAZ domain and the active site [35].

Genetic studies revealed that Dicer is essential for mammalian development, as Dicer-deficient mice die at the embryonic stage [50, 51]. However, the *DCR*-1 gene can be disrupted in mouse embryonic stem (ES) cells by conditional gene targeting. The generated Dicer-null ES cells are viable, despite being completely defective in the generation of miRNAs, and display severe defects in differentiation both in vitro and in vivo [52]. Similar conditional inactivation of the Dicer gene in ES cell lines compromised proliferation as well as miRNA maturation, possibly rationalizing the phenotype

observed in Dicer-null animals [53]. Epigenetic silencing of centromeric repeat sequences [52, 53] and expression of homologous small dsRNAs [52] were also markedly reduced in Dicer-null ES cells. Re-expression of Dicer in knockout cells rescued these phenotypes [52]. These results suggest the involvement of Dicer in multiple fundamental biological processes in mammals, ranging from stem cell differentiation to maintenance of centromeric heterochromatin structure and centromeric silencing [52].

It is relevant to note that Dicer activity is potently stimulated by limited proteolysis induced by low concentrations of proteinase K in vitro [47, 48], indicating the presence of intrinsic regulatory domains of Dicer activity. As recently reported, cellular proteins interacting with Dicer such as Ago2 [54], fragile X mental retardation protein (FMRP) [55], TRBP [56, 57], and the protein kinase R (PKR)-activating enzyme (PACT) [58] may also represent key regulators of Dicer activity. In addition, Dicer was recently shown to be a part of an effector miRNP [24], thereby coupling the initiation and effector steps of miRNA-guided RNA silencing.

TRBP

TRBP was identified and characterized in 1991 as a cellular factor acting in synergy with the viral Tat protein in the transactivation of the long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1), leading to viral gene transcription [59]. TRBP exists mainly in two different isoforms: TRBP1 and TRBP2 [60], which possess three dsRBDs and a basic C-terminus, coexist in the cell and are encoded by two alternatively initiated isoforms of mRNA that differ at their 5′ ends. TRBP2 is 21 amino acids longer than TRBP1 [60–62]. TRBP has also been shown to bind Tax of human T-cell leukemia virus 1, although this interaction inhibits the transactivating activity of Tax [63]. Another function of TRBP is the inhibition of the interferon-induced dsRNA-regulated PKR [64].

Recently, TRBP has been reported to play a role in miRNA-guided RNA silencing. TRBP was identified by proteomic analysis of immunoprecipitates prepared from HEK 293-derived stable cell lines expressing a Flag-tagged Dicer [56]. Further analyses revealed the association of Dicer-TRBP with Ago2 and the requirement of TRBP for the recruitment of Ago2 to the siRNA bound by Dicer. TRBP was shown to facilitate the cleavage of pre-miRNAs in vitro and optimize RNA silencing mediated by siRNAs and endogenous miRNAs [57]. These results support a role for TRBP, the first Dicer-interacting protein identified since Ago2, in assisting Dicer function in a pre-miRNA processing complex and contributing to RISC assembly by the recruitment of Ago2 to the miRNA.

A Dicer interaction with the *Drosophila* homologue of human TRBP, Loquacious (Loqs), which share 34% identity at the amino acid level, was also observed by two independent groups [65, 66]. As for human TRBP, Loqs was required for normal processing of pre-miRNAs by Dicer-1 [65, 66] and for efficient miRNA-mediated silencing in various contexts [65]. Thus, every known fly RNase III is paired with a dsRBD-containing protein that facilitates its function in small RNA biogenesis.

R2D2

The siRNA-generating complex purified from *Drosophila* S2 cells consists of two stoichiometric subunits: Dicer-2 and R2D2 [67]. R2D2, which was named so because it contains two dsRNA-binding domains (R2) and is associated with DCR-2 (D2) in *Drosophila* [67], is homologous to *C elegans* RDE-4 [68, 69]. The Dicer-2/R2D2 complex, but not Dicer-2 alone, binds to siRNA and enhances sequence-specific mRNA degradation mediated by the RISC. R2D2 has been shown to act as a biosensor for detecting thermodynamic differences of base pairing at the extremities of an siRNA [70]. Thus, in concert with Dicer-2, R2D2 determines which siRNA strand will be incorporated into the RISC and may also discriminate an impostor siRNA [71, 72]. These results indicate that R2D2 bridges the initiation and effector steps of the *Drosophila* RNAi pathway by facilitating siRNA passage from Dicer to RISC. Whether a similar mechanism is operating in humans remains to be investigated.

RISC and miRNPs

The miRNA or siRNA generated by Dicer is loaded into an effector miRNP or siRNP complex, respectively, and guides it for the recognition and regulation of the mRNA target. The mRNA specifically recognized by the RNP complexes will initially be either cleaved or translationally repressed, depending on whether the guide:mRNA pairing is perfect or not [25]. In humans, mRNA regulation by miRNAs is believed to consist mainly in translational repression, although a recent study reported that miRNAs downregulate a greater number of transcripts than previously thought [73]. Yekta et al [74] demonstrated that miR-196 shows perfect complementarity (presence of a single G:U wobble) with HOXB8 mRNA and directs its cleavage in mouse embryos. Genes for miR-196 map to homeobox (HOX) clusters, which encode transcription factors crucial for the developmental program in animals. Bagga et al [75] observed that the let-7 miRNA induces degradation of its target, lin-41, in *C elegans*. Furthermore, they observed that the level of the lin-4 miRNA targets, lin-14 and lin-28, is decreased in response to lin-4 expression. These observations suggest that mRNAs containing partial miRNA complementary sites may not only be subjected to translational repression, but also be targeted for degradation in vivo.

Initial studies on the RISC reported the existence of a large ($\sim$ 150 kDa to $\sim$ 500 kDa) multiprotein RNP complex exhibiting sequence-specific nuclease activity [54, 76, 77]. Small RNAs of ~21 to 25 nt were found to copurify with the RISC isolated from *Drosophila* S2 cells [76], a characteristic shared by RISC complexes from other species [78]. A study by Pham et al [78] provided the first glimpse of the mechanism involved in RISC assembly. The authors proposed a three-step model for RISC formation in *Drosophila*. Isolation of three distinct complexes, named R1, R2, and R3, by native gel electrophoresis showed that siRNA binding to Dicer-2 is

responsible for R1 formation. R1 is probably the ∼360 kDa complex described as the RISC [77]. R1 serves as a precursor to form both the R2 and R3 complexes. R3 is a large ATP-enhanced complex that contains unwound siRNAs, cofractionates with known RNAi factors, binds and cleaves targeted mRNAs in a cognate siRNA-dependent manner [78].

Recently, three studies published in the same issue of Cell [24, 79, 80] provided additional insights on the composition, assembly, and function of the RISC. Gregory et al [24] showed that the human RISC is composed of at least three proteins: Dicer, TRBP, and Ago2. Recently, the dsRBD protein PACT was also found to be associated with Dicer, hAgo2, and TRBP in a ∼500 kDa complex and to function as a component of the RISC [58]. At first, an ATP-dependent helicase was proposed to separate the two siRNA strands, one of which was thought to bind to Ago2. However, a recent consensus model suggests that Ago2 directly receives the double-stranded siRNA and cleaves the siRNA passenger strand instead, thereby liberating the ss guide for mediating cleavage or repression of the RNA target [24, 79, 80]. In contrast, passenger-strand cleavage is not important for the incorporation of miRNAs that are derived from mismatched duplexes, suggesting that this mechanism may not apply to endogenous miRNAs in humans.

In 2002, Mourelatos et al [81] reported the identification and characterization of a miRNP complex showing high similarity with the RISC. The authors isolated a wide range of different miRNAs forming a complex with three major proteins: Gemin3, Gemin4, and EIF2C2 (hAgo2). Gemin3, a 105 kDa DEAD-box putative helicase, may be involved in unwinding the double-stranded miRNA and releasing the miRNA* strand for recognition of the target.

P-/GW-bodies

Where does the miRNP-mediated mRNA regulation or cleavage occur in the cell? Recent studies revealed the existence of specific cytoplasmic foci, referred to as processing (P-bodies) [82, 83] or GW182-containing bodies (GW-bodies) [84]. The GW bodies, which were named so because they contain the GW182 RNA-binding protein, are enriched in proteins that are involved in mRNA degradation [85]. Liu et al [82] demonstrated the localization of Ago proteins into mammalian P-bodies. In fact, Ago proteins were found to interact with GW182 [86]. Silencing of GW182 or mutations that prevented Ago proteins from localizing in P-/GW-bodies impaired translational repression of mRNAs [86]. The presence of exogenous siRNAs was also detected in these bodies [87]. These studies support a functional link between cytoplasmic P-/GW-bodies and mRNA translation repression mediated by miRNAs.

These cytoplasmic P-/GW-bodies may not be the only sites of mRNA degradation in the cell. Two independent groups also detected a RISC-like activity in the nucleus of cultured mammalian cells [88, 89]. It is tempting to speculate that the nuclear effector complex mediating this activity may be closely related to the RNA-induced initiation of transcriptional gene silencing (RITS) complex found in *S pombe* [90].

Ago2

Ago2 is a member of the PAZ and Piwi domain (PPD) protein family, which is composed of highly basic proteins that are present in metazoans and fungi, but not in the budding yeast *Saccharomyces cerevisiae* [91, 92]. Eight members of the Ago family are expressed in humans [93], and the isoforms Ago1 to 4 are closely related. All four can bind siRNAs and miRNAs, but only Ago2 is present in an mRNA-cleavage competent RISC [94, 95]. Several paralogues of Ago proteins are found across the kingdoms and their number varies from 1 in *S pombe* [96] to more than 20 in *C elegans* [43, 97].

Structural studies have provided key insights into the mechanism of RNAi. Ago2 is composed of a central PAZ domain and a C-terminal PIWI domain. The nuclear magnetic resonance solution structure of the *Drosophila* Ago1 PAZ domain bound to RNA was resolved recently [98, 99]. The structure consists of a left-handed, six-stranded β-barrel capped at one end by two α-helices and wrapped on one side by a distinctive appendage, which comprises a long β-hairpin and a short α-helix. Combined structural and binding studies of the PAZ domain indicated that it provides a binding pocket for the 3′ protruding ends of siRNAs [98–101].

Structural studies revealed that the PIWI domain consists of 5-stranded β-sheets surrounded by three helices [102] and mediates binding of the ss RNA 5′ end [103–105]. The structure of *Archaeoglobus fulgidus* PIWI domain in complex with an siRNA-like duplex, which mimics the 5′ end of a guide RNA strand bound to an overhanging target mRNA, has been solved. This study revealed the presence of a highly conserved metal-binding site that anchors the 5′ nt of the guide RNA [105]. Structural studies also determined PIWI as the catalytic domain for the nuclease activity of Ago2, given its resemblance to RNase H [95, 102, 104], in terms of structure and activity; like RNase H, Ago2 activity is dependent on divalent cations such as Mg^{2+} or Mn^{2+} [106]. The PIWI domain and RNase H also share a DDE motif, similar to those present at the catalytic center of integrase proteins [107].

Structural information from *Pyrococcus furiosus* Ago [102], together with the demonstration that Ago2 is the core slicing machine of the human RISC [95, 108], provided strong evidences suggesting that the PIWI domain may be responsible for mediating this "slicer" activity. This possibility was further supported by the observed inhibition of target mRNA cleavage activity upon deletion of the DDE motif of hAgo2 [95]. In siRNA-guided RNA silencing, Ago2 cleaves, in an ATP-independent manner, the phosphodiester backbone of the target mRNA between nucleotides 10 and 11, as calculated from the RNA guide 5′ end [80, 94, 109].

The mechanism of translation repression mediated by Ago proteins is still unclear, although recent evidences suggest the possibility that some Ago-containing complexes may repress translation in P-bodies [110, 111].

FMRP

In human, loss-of-function mutations in the *FMR1* (fragile mental retardation 1) gene product FMRP is the cause of the most common mental retardation, the fragile X syndrome

[112, 113]. An expansion of the CGG repeat in the 5′NTR of *FRM1* is associated with DNA methylation problems of both the CpG island and the CGG repeat itself, resulting in an inhibition of transcription and translation [114, 115].

FMRP is a cytoplasmic RNA-binding protein found to be associated with polyribosomes as part of an mRNA ribonucleoprotein (mRNP) complex, suggesting a role for FMRP in mRNA translation regulation [112]. In fact, this protein of 632 amino acids, containing two K-homology (KH) domains and an RGG box, acts as a negative regulator of translation in vitro and in vivo [116–118]. A relationship between FMRP and the RNAi pathway was unveiled by the copurification of dFMR1 with the *Drosophila* RISC, which also contains Ago2 and the vasa intronic gene (VIG) [119]. Similarly, Ishizuka et al [55] used a tandem affinity purification approach to isolate an RNP complex that contains dFMR1, Ago2, the RNA helicase Dmp68, and the ribosomal proteins L5/5S RNA and L11. Ishizuka's group demonstrated that dFMR1 is a component of the RISC effector complex and is associated with Dicer and Ago2 [55]. Knockdown of dFMR1 by introduction of dFMR1 dsRNA had only mild effects on the efficiency of RNAi [55, 119].

The work of Jin et al [120] suggested that FMRP could interact with miRNAs, Dicer, and Ago1 in mammalian cells in vivo, raising the possibility that FMRP could use miRNAs to regulate translation of specific mRNAs. Indeed, a recent study from our laboratory showed that human FMRP can act as an miRNA acceptor protein for Dicer and facilitate the assembly of miRNAs on specific target RNA sequences [121]. This activity appears to be mediated by the KH domains. In this study, the requirement of FMRP for efficient RNAi in vivo was unveiled by reporter gene silencing assays using various small RNA inducers, which also supported its involvement in an ss siRNP effector complex in mammalian cells. These results defined a possible role for FMRP in miRNA-guided RNA silencing and provided further insight into the molecular defects in patients with the fragile X syndrome.

VIG

The VIG protein has been shown to be associated with the *Drosophila* RISC [119]. An evolutionarily conserved protein expressed in *C elegans*, *A thaliana*, *S pombe*, and mammals, VIG has no recognizable protein domains other than an RGG box, a motif that is known to bind RNA. Although no function has been assigned to VIG, its human homologue, plasminogen activator inhibitor (PAI)-RBP-1, was originally identified as a protein having an affinity for AU-rich elements (ARE) located in the 3′NTR of PAI RNA and regulating its stability [122]. The authors also demonstrated the importance of Dicer and miR-16, a human miRNA containing a sequence complementary to ARE, in conferring instability to ARE-containing mRNAs. This suggests an interesting connection between the components of the miRNA-guided RNA silencing pathway and regulation of the stability of mRNAs containing AREs, which are known to act *in cis* to regulate rapid turnover of unstable mRNAs [123] in their 3′NTR. The exact role of VIG in that context remains to be investigated.

Tudor-SN

The staphylococcal nuclease Tudor (Tudor-SN) has been identified as a component of the RISC in *C elegans, D melanogaster*, and humans [124]. Tudor-SN contains five staphylococcal/micrococcal domains and a tudor domain. At first, Tudor-SN was suspected to be the nuclease responsible for the RISC-mediated mRNA target cleavage. However, studies demonstrating that the nuclease activity of the RISC is Mg^{2+}-dependent [106] and produces 5′-phosphomonoester ends [125] did not support this hypothesis, as Tudor-SN is rather a Ca^{2+}-dependant nuclease that generates 3′-phosphomono- and dinucleotides from DNA or RNA substrates [126].

Recently, a novel relationship was established between Tudor-SN and adenosine deaminases that act on RNA (ADARs). Members of the ADAR family exhibit affinity with dsRNAs and mediate an RNA editing reaction that substitutes adenosine (A) residues by inosines (I) in cellular mRNAs or other dsRNA targets [127]. Scadden [128] showed that Tudor-SN specifically interacts with and promotes the cleavage of model hyper-edited dsRNA substrates containing multiple IU and UI pairs. Yang et al [129] have recently reported that the edition of pri-miR-142 resulted in the suppression of its processing by Drosha, and was instead degraded by Tudor-SN. Similarly, pre-miRNAs have also been shown to be edited by ADARs [130]. ADAR-induced modification of pri- and pre-miRNA sequences may also contribute to diversifying and influencing the genetic control mediated by miRNAs. For example, structural changes induced by A-to-I edition of pri- and pre-miRNAs may hamper their recognition and processing by the dsRNA-cleaving Drosha and Dicer RNases [131–133]. These studies reveal a new function for RNA editing in the control of miRNA biogenesis.

RITS complex

In the fission yeast *S pombe*, dsRNA arising from centromeric repeats targets the formation and maintenance of centromere function through RNAi-mediated histone H3 lysine-9 (K9) methylation [90]. This is accomplished by the effector complex RITS, which contains the proteins Ago1, Chp1, and Tas3, in addition to small RNAs [96] generated by Dicer [134]. Homologous to centromeric repeats [135], these small RNAs appear to guide the RITS components to heterochromatic regions, such as the centromeres, the mating-type region, and the telomeres [136, 137]. Upon centromeric binding, RITS promotes Clr4-mediated methylation of histone H3 K9, recruitment of Swi6 [138], and formation of heterochromatin [96]. Recently, a study concluded that Dicer and the RNAi machinery were involved in the formation of heterochromatin in higher vertebrate cells, as suggested previously [134]. The discovery of the effector RITS complex supports a nuclear function for small RNAs derived from Dicer.

IDENTIFICATION OF miRNAs AND THEIR TARGETS

As the major protein components of the miRNA-guided RNA silencing pathway are being identified and characterized, hundreds of new miRNAs are being discovered in several different species. The fact that the interaction between miRNAs and the mRNA targets they regulate is based mainly on partial, rather than perfect, complementarity renders target idenfication rather arduous. However, improvement of our understanding of the determinants governing mRNA recognition by miRNAs has allowed the development of several predictive bioinformatic tools. The growing number of miRNA targets and functions, as revealed by various experimental approaches, let us foresee the importance and complexity of the gene regulatory network utilizing miRNAs.

Identification of miRNAs

Almost 8 years after the discovery of the ncRNA lin-4, known for its crucial role in developmental timing in *C elegans* [3, 5], three independent groups defined miRNAs as a novel family of small (~22 nt) regulatory RNAs that are diverse in sequence and expression patterns, and evolutionarily widespread [10–12]. The authors used different strategies to identify new miRNAs from various species. miRNAs showing features reminiscent of Dicer cleavage can be cloned by reverse transcription-polymerase chain reaction (RT-PCR) on size-fractionated RNA populations. If their sequences are known or predicted, and if they are abundant enough, miRNAs can be detected by Northern blot analysis.

Rapid and large-scale identification of miRNAs prompted experts in the field to establish guidelines for miRNA annotation and institute different criteria, based on expression and biogenesis, for an RNA to be considered as an miRNA [139]. First, a 22 nt RNA transcript must be detected by Northern blot analysis. Second, the RNA transcript must be detected in a cDNA library prepared from size-fractionated RNA samples. Third, bioinformatic analyses must predict a hairpin-loop structure encoded in the genome and the sequence has to be located on one arm of this structure with a lowest free energy. The hairpin should have small bulges and approximately 60–80 nt in length. Phylogenetic conservation among species represents another important feature which, however, excludes miRNAs that have either disappeared, appeared, or evolved during the course of evolution.

Computational algorithms designed to identify hairpin-loop structures and sequence conservations across species are very useful, especially for less abundant or tissue-specific miRNAs. These small RNAs can be regrouped into families, based on the sequence of their 5′ region [139]. One computational algorithm that has been developed and tested with *C elegans*, miRScan, uses different characteristics to identify miRNA genes. It has been designed to find conserved sequences upstream and downstream of the miRNA foldback, identify specific adjacent sequences that can be involved in miRNA transcription or processing, and determine the location of cotranscribed miRNAs in orthologous host genes [140].

miRBase is the new home of the miRNA data on the web, accessible at the following address: http://microrna.sanger.ac.uk/ [141]. It provides information previously accessible from the miRNA registry [142]. As of May 2006 (release 8.1), there were 462 human miRNA sequences among 3963 entries.

To date, miRNA genes constitute about 2% of the predicted genes in mammals. They may be constitutively or developmentally regulated and expressed at various levels in different tissues. Recent estimates suggest that between 30% and 50% of the genes may be regulated by miRNAs [144, 145]. This raises the possibility that all the cellular pathways may be governed by miRNAs. However, the question remains: which mRNAs are subjected to miRNA regulation?

Identification of miRNA targets

Identification of miRNA targets is a key step in understanding the biological function of miRNAs. However, the progress of this work is hampered by the mode of mRNA recognition by the regulatory miRNAs itself, which is based on imperfect sequence complementarity [25]. Characterization of a few experimentally validated miRNA:mRNA interactions allowed to establish a context in which this interaction is favored and helped to develop very useful bioinformatic approaches to identify them. Initial studies indicate that a given miRNA may regulate several different mRNAs and that, conversely, a specific mRNA can be regulated by more than one miRNAs.

Several algorithms currently available on the web, such as TargetScan (http://genes.mit.edu/targetscan/) [146], Miranda (http://www.microrna.org) [147], and DIANA-microT (http://www.diana.pcbi.upenn.edu/) [148], combine different parameters of the sequence requirements for miRNA:mRNA binding as predictive methods to identify targets. These computational tools are designed to scan the 3′NTR of mRNA targets, to search for the miRNA seed and to determine the free energy of the interaction. They can also take into account the phylogenetic conservation and the presence of more than one miRNA binding site in a given 3′NTR. Because each of these methods uses different miRNA:mRNA target predictive determinants, the results obtained may differ from one to another. Nevertheless, these bioinformatic tools are crucial in providing initial cues as to the possible mRNA targets regulated by specific miRNAs. They also offer a certain basis for initiating experimental validation on miRNA:mRNA target pair of interest. In turn, a better comprehension of the interaction between miRNAs and their targets will permit the improvement of these predictive methods.

Vella et al [143, 149] studied the well-characterized let-7:lin-41 interaction to better understand the architecture and requirements of miRNA:mRNA target recognition. Although the lin-41 mRNA target bears six putative let-7 miRNA binding sites, only two of them appear to be necessary for lin-41 regulation (see Figure 3). These two sites are separated by a 27 nt sequence. Generally, the miRNA seed consists in a perfect pairing between miRNA nucleotides 2 to 8 with a

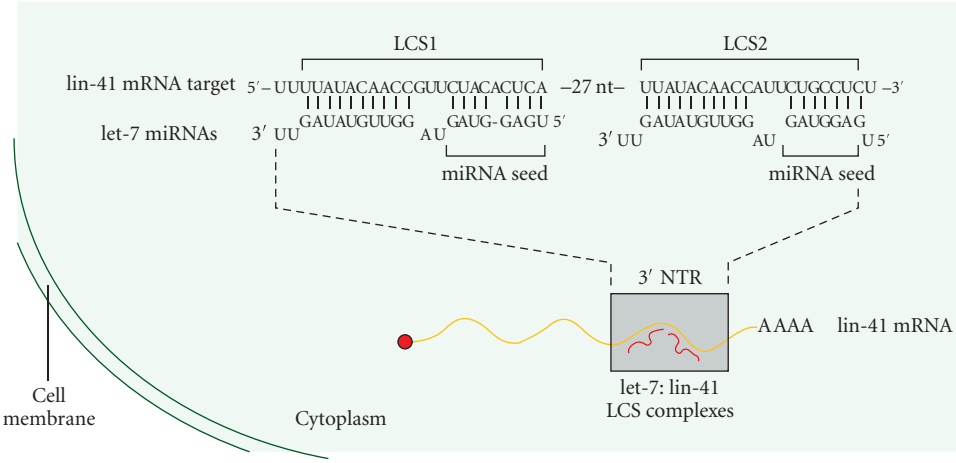

Figure 3: Recognition of the lin-41 mRNA by let-7 in *C elegans*. The lin-41 3′NTR contains two let-7 complementary sites (LCS) separated by a 27 nt sequence that seems to be important in target recognition [143]. The miRNA seed consists in a perfect pairing of nucleotides 2 to 8 of the miRNA.

sequence located in the 3′NTR of its mRNA target. Although pairing of the 3′ region of an miRNA seems to be less important, it may compensate a weaker binding of the 5′ region. The authors also observed that lin-41 regulation by let-7 was lost upon substitution of the intervening 27 nt sequence by another [143]. This suggests that miRNA:mRNA interactions do not rely solely on the regions of complementarity and may be more complex than previously thought.

In spite of the difficulties to identify miRNA targets, several groups have found their way to assign a biological function to some miRNAs. Hematopoietic cell lineages derived from mouse bone marrow express specific miRNAs that regulate differentiation. Chen et al [150] analyzed three miRNAs, miR-181, miR-223, and miR-142 that were differentially or preferentially expressed in hematopoietic cells. They observed that overexpression of these miRNAs in undifferentiated progenitor cells derived from mouse bone marrow altered lineage differentiation. They further analyzed the effects of miR-181 in vivo by infecting mouse Lin^- bone marrow cells with a viral vector expressing this miRNA and observed that mice expressing miR-181 had a substantial increase in B-lymphoid ($CD19^+$) cells. Lim et al [73] used a microarray approach to identify miRNA targets after overexpression of known miRNAs. They found that 174 genes were downregulated following overexpression of miR-124, an miRNA preferentially expressed in the brain, in HeLa cells. Incidentally, the target genes were expressed at low levels in the brain. Thus, the expression of miR-124 in HeLa cells caused a shift in gene expression profile towards that of the brain. Using the same approach, expression in HeLa cells of miR-1, expressed in muscle, shifted the expression profile of HeLa cells towards that of the muscle.

BIOLOGICAL FUNCTION OF miRNAs

As experimental evidences are accumulating on how miRNAs recognize and regulate specific mRNA targets, we are beginning to understand the exact function of each miRNA as well as the cellular processes they are regulating. Information pertaining to the biological function of miRNAs in different species, which is the subject of this section, is summarized in Table 2.

miRNAs and development

Developmental studies of the nematode *C elegans* led to the discovery of the first small noncoding regulatory RNA, lin-4. *C elegans* proceeds through four larval development stages termed L1 to L4. Transition from one stage to the next is dictated by temporally regulated heterochronic genes, which are involved in developmental regulatory cascades. Wightman et al [4] first reported that short repetitive sequences in the 3′NTR of the heterochronic gene *lin-14* were negative regulatory elements of Lin-14 expression. The observed increase in Lin-14 protein synthesis associated with two gain-of-function mutations in the 3′NTR of lin-14 mRNA [4] was instrumental for this discovery. More than two years later, Lee et al [3] identified two lin-4 transcripts, one of 61 nt and another of 21 nt. Furthermore, they observed that the lin-4 smaller transcript was complementary to seven repeated sequences in lin-14 3′NTR, identified previously by Wightman et al [4]. These findings suggested that lin-4 could regulate lin-14 translation via an antisense RNA:mRNA interaction [3]. Recently, Boehm and Slack [169] found that lin-4 and lin-14 expression control life span through adulthood, since lin-4 loss-of-function mutant is associated with a shorter life span as compared to wild-type nematodes, whereas overexpression of lin-4 prolonged it. They also noted that animals carrying a temperature-sensitive loss-of-function mutation in *lin-14* had a 31% longer life span than wild-type, which is consistent with the phenotype observed with *lin*-4 [169].

A second small ncRNA, let-7, was later identified and found also to regulate the transition from late larval L4 to adult stage through the regulation of heterochronic genes

Table 2: Biological functions of miRNAs in different species.

Species	Expression	miRNA	mRNA target	Validation (Expt/Pred)	Mode of regulation	Process regulated	References
Schizosaccharomyces pombe	—	12 different small RNAs	Centromeric regions	Expt	H3 K9 DNA methylation	Maintenance of heterochromatin	[135]
Caenorhabditis elegans	—	lin-4	lin-14 lin-28	Expt	Translational repression/ mRNA cleavage	Control of the developmental stages L1-L3	[3, 4, 75, 151]
	—	let-7	hbl-1 lin-41 daf-12 ras	Expt	Translational repression/ mRNA cleavage	Control of the last developmental stage L4	[75, 149, 152–156]
	—	miR-48 miR-84 miR-241	hbl-1	Expt	Translational repression	Control of the L2 to L3 transition	[157]
Drosophila melanogaster	—	bantam	hid	Expt	Translational repression	Inhibition of apoptosis	[158]
	—	miR-2 miR-6 miR-11 miR-13 miR-308	ND	Expt	ND	Inhibition of apoptosis	[159]
Mus musculus	Hematopoietic cells	miR-142 miR-181 miR-223	ND	Expt	ND	Hematopoietic cell differentiation	[150]
	Mouse embryo < day 7	miR-196	HOXB8	Expt	mRNA cleavage	Control of the developmental program	[74]
Homo sapiens	Brain	miR-124	ND	Expt	ND	Brain-specific gene expression	[73]
	Muscle	miR-1	ND	Expt	ND	Muscle-specific gene expression	[73]
	Overexpression in brain tumor glioblastoma	miR-21	ND	Expt	ND	Inhibition of apoptosis	[160]
	Downregulation in CLL	miR-15a miR-16-1	Bcl2	Expt	Translational repression	Induction of apoptosis	[161, 162]
	Downregulation in numerous cancer cultured cells	miR-143 miR-145	ND	Expt	ND	Oncogenesis	[163]
	Overexpression in BL patient	miR-155	ND	Expt	ND	Oncogenesis	[164]
	Overexpression in B-cells lymphomas and lung cancer cell lines	miR-17-92 cluster	ND	Expt	ND	Oncogenesis	[165]

Table 2: Continued.

HeLa, 293T	miR-32	ORF2 in the 3′NTR of all remaining PFV-1 mRNAs	Expt	ND	Restriction of PFV-1 accumulation	[166]
Liver-specific expression	miR-122	5′ noncoding region of the HCV genome	Expt	ND	Facilitation of HCV replication	[167]
T cells	miR-29a miR-29b	*nef*	Pred	ND	Inhibition of HIV-1 replication	[168]
T cells	miR-149	*vpr*	Pred	ND	Control of the cell cycle arrest in G2	[168]
T cells	miR-378	*env*	Pred	ND	Control of the virus assembly	[168]
T cells	miR-324-5p	*vif*	Pred	ND	Control of viral particle production	[168]

BL: Burkitt lymphoma; CLL: chronic lymphocytic leukemias; Expt: experimental; HCV: hepatitis C virus; HIV-1: human immunodeficiency virus type 1; ND: not determined; PFV-1: primate foamy virus type 1; Pred: predicted.

in *C elegans* [152]. Northern blot analyses revealed that the miRNA let-7 is expressed in a wide range of species, including worm, fly, and human, as opposed to lin-4, and seems to regulate late developmental transition in different species [153]. Three let-7 miRNA family members, miR-48, miR-84, and miR-241, were identified on the basis of sequence identity of 8 consecutive nucleotides in their 5′ region [11, 170]. let-7 regulates lin-41, hbl-1, and daf-12 [149, 152–155]. The other members of the let-7 family appear to regulate hbl-1 in the L2 to L3 transition [157]. let-7 also appears to be important for zebrafish embryo development, since injection of a synthetic let-7 miRNA duplex into zebrafish zygotes causes severe growth defect [171]. Embryos of a maternal-zygotic zebrafish Dicer mutant that were unable to process pre-miRNA into miRNA showed abnormal brain morphogenesis [172]. This brain defect was rescued by the injection of a preprocessed, mature miRNA, miR-430. The miRNA expression profile of zebrafish embryos is highly tissue-specific during segmentation and later stages, but not in early development, suggesting that miRNAs may play a more prominent role in differentiation or maintenance of tissue identity, rather than in directing tissue fate [173].

miRNAs have also a major role in developmental regulation in fly. This conclusion came from miRNA loss-of-function analyses using 2′ O-methyl (Me) antisense oligoribonucleotides in *Drosophila* embryos [159]. In these analyses, depletion of as many as 25 of 47 miRNAs expressed in early development caused a severe developmental phenotype. In situ hybridization analyses, using probes recognizing 38 different miRNAs in *Drosophila* embryos, indicated that the expression profile of most of them is comparable to their vertebrate counterparts [174], suggesting an evolutionarily conserved role for miRNAs in development.

Recently, two groups independently reported the cloning of the mouse and chicken homologues of *C elegans lin*-41 [175, 176]. They found that *mlin-41* and *clin-41* are implicated in limb development. Bioinformatic analyses confirmed the presence of let-7 binding sites in the 3′NTR of these two genes. In mice, targeted disruption of the *Dicer*1 gene was lethal in early development, indicating that Dicer function is essential for proper development in mammals [50]. Harfe et al [177] used an inducible inactivation system of *Dicer1* to study its importance in late development in mice. In this model, depletion of Dicer led to a severe defect in limb formation.

miRNAs and heterochromatin

The RNAi pathway was also reported to play a role in nuclear events such as genome rearrangement [178], gene inhibition [90, 134, 179], and chromosome segregation [134], supporting the idea that the genome integrity itself is preserved by small regulatory RNAs. In a model proposed by Noma et al [136], dsRNA transcripts are cleaved by Dicer to produce siRNAs, which are incorporated into the RITS complex and guide it to heterochromatic regions, probably through interactions with DNA or native RNA transcripts. Once localized at the siRNA homologous target sequence, the RITS complex recruits the Clr4 methyltransferase that catalyzes methylation of histone H3 at lysine 9. This creates binding sites for the heterochromatin protein Swi6 which, in turn, leads to the recruitment of additional Clr4 and further H3-Lys9

methylation of adjacent nucleosomes. These modifications allow the binding of RITS in a Dicer-independant manner via the chromodomain of Chp1 and the maintenance of gene repression at the transcriptional level. Although this process was first documented in *S pombe* [96, 136, 180], recent reports indicate the occurrence of a similar transcriptional gene silencing phenomenon in the nucleus of human cells [181, 182]. Altogether, these studies reveal a key role for Dicer-derived small RNAs in guiding the RITS complex and regulating the transcriptional and posttranscriptional status of host gene expression.

miRNAs in cell growth and apoptosis

Cell growth and programmed cell death are important processes implicated in both development and differentiation. The *bantam* gene identified in *Drosophila* was first discovered on the basis of its effect on tissue growth: tissues were larger when *bantam* was overexpressed and smaller when *bantam* expression was suppressed. Although smaller, the flies were proportional and did not exhibit patterning defects [158]. Later, the same group determined that the *bantam* gene encoded for an miRNA, not for a protein, that controlled the proapoptotic gene *hid*. Thus, *bantam* promotes proliferation while inhibiting apoptosis [183]. Additional miRNAs involved in the regulation of pro-apoptotic genes in *Drosophila* were discovered in loss-of-function experiments. In that context, a family of miRNAs comprising miR-2, miR-6, miR-11, miR-13, and miR-308 has been found to be required for suppression of embryonic apoptosis [159].

Chang et al [184] used a library of miRNA antisense oligonucleotides bearing 2′ O-Me modifications to inhibit miRNA function in HeLa cells. Monitoring changes in cell growth and apoptosis, they identified several important regulatory miRNAs. In the highly malignant human brain tumor glioblastoma, miR-21 was strongly overexpressed. When miR-21 was knocked down in glioblastoma cultured cells, caspases were activated, causing an increase in cell apoptosis [160]. This suggests a role for miR-21 as a suppressor of apoptosis in this malignant tumor [160]. In chronic lymphocytic leukemias (CLL), the antiapoptotic protein B-cell lymphoma 2 (Bcl2) is overexpressed [161]. Interestingly, frequent deletions and downregulation of the miR-15 and miR-16 genes at the chromosome locus 13q14 are observed in the majority of CLLs [162]. These findings suggest a role for miR-15a and miR16-1 as repressors of Bcl2 expression and possible inducers of apoptosis [161].

RELATIONSHIP BETWEEN miRNAs AND DISEASES

Given their recognized importance in gene regulation, a link between miRNAs and several major diseases is expected. For example, defects in miRNA-mediated regulation of mRNA translation may lead to overexpression of specific proteins, which accumulation may cause diseases. In fact, intriguing connections between miRNAs and diseases, such as cancer and viral infections, are emerging.

miRNAs and cancer

A recent study reported that human miRNA genes are frequently located at fragile sites and genomic regions involved in cancer [185]. Indeed, Calin et al [162] observed frequent deletions and downregulation of *miR*-15 and *miR*-16 genes at 13q14 in CLL. These miRNAs have been shown to negatively regulate the antiapoptotic Bcl2 protein at the posttranscriptional level [161]. BCL2 repression by these miRNAs induced apoptopsis in a leukemic cell line model [161], thereby providing a link between the absence of miR-15/miR-16 and leukemia.

Northern blot analyses showed that miR-143 and miR-145 expression is downregulated in various human cell lines derived from breast, prostate, cervical, lymphoid cancers, and, particularly, colorectal tumors [163]. Potential targets of these miRNAs have been previously implicated in oncogenesis [163].

A relationship between miRNAs and Burkitt lymphoma (BL) has been suggested. miR-155 is encoded within nucleotides 241–262 of the *BIC* gene, which is located on chromosome 21. Both the *BIC* and *miR*-155 genes are overexpressed in some BL patients, but not in all BL cases [164, 186]. Abnormal miRNA expression may thus contribute to the transformation of B cells [164].

Another miRNA cluster, miR-17-92, is often overexpressed in tumor samples from B-cell lymphomas when compared to normal cell lines [165]. This cluster is present in an amplified DNA region encoding for the ORF *c13orf25*. Alignment of this ORF between mouse and human indicates that the polycistron and its immediate flanking sequences only are conserved. The c13orf25 transcript contains seven pre-miRNAs encoding for miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-20, miR-19b-1, and miR-92-1. Using a microarray analysis of 191 mature miRNAs, five miRNAs from the cluster were found to be highly expressed in B-cell lymphomas, in correlation with an increased expression of c13orf25 [165]. These studies revealed that the miR-17-92 cluster can act as a potential human oncogene, and was referred as oncomiR-1 by Hammond and colleagues [165].

Another group subsequently reported that the miR-17-92 cluster was overexpressed in human lung cancer cell lines [187]. When analyzing the subcellular localization of the c13orf25 transcript, they observed a nuclear localization, restricting its cytoplasmic localization for translation. This suggested that this transcript can act as a vehicle for the expression of the miR-17-92 cluster. Its predicted targets include the tumor suppressor genes *PTEN* and *RB2* [146]. In a mouse B-cell lymphoma model, the overexpression of miR-17-92 accelerates tumor formation induced by the product of the *MYC* gene [165], which encodes an important transcription factor that regulates cell proliferation, growth, and apoptosis. Modification of *MYC* expression is a commonly observed deregulation leading to tumorigenesis. O'Donnell et al showed that c-myc, through binding to sequences near the miR-17-92 cluster genomic locus, activates miR-17-92 expression [188]. Among the predicted targets of the miR-17-92 cluster is the transcription factor E2F1, which appears to be negatively regulated by miR-17-5p and miR-20a [146].

E2F1 is a cell cycle promoter induced upon c-myc expression. Conversely, c-myc expression is induced by E2F1 [189–191]. Therefore, a balance between the gene regulatory processes involving miRNAs and transcription factors may contribute to finely tune E2F1 expression and to generate a tightly controlled proliferative signal [188].

The let-7 miRNA also seems to be involved in cancer pathogenesis. Calin et al reported that *let*-7 genes are deleted in many cancers [185]. Moreover, a reduction in let-7 expression has been observed in samples of human lung cancers or cancer cell lines. Patients associated with a reduced let-7 expression had the worst prognosis after a potentially curative resection [192]. It is relevant to note that the overexpression of let-7 in lung cancer cells inhibits growth in vitro [192]. In *C elegans*, let-60, the ortholog of the human oncogene RAS, was found to contain eight putative let-7 binding sites in its 3′NTR [156]. As for the three human *RAS* genes, they also contain multiple let-7 binding sites, allowing let-7 to regulate RAS expression. Evidences of a downregulatory role for let-7 in RAS expression came from experiments using reporter genes fused to the 3′NTR of NRAS and KRAS [156]. Introduction of let-7a inhibitors relieved this repression [156]. In this study, miRNA microarray analyses of 21 different samples from lung cancer patients revealed that the expression of the *let*-7 gene family was reduced, in association with an increased expression of RAS protein. These data suggest a role for the let-7 miRNA family in the regulation of RAS during development of lung cancer [156].

Deregulated expression of protein components of the miRNA biosynthetic pathway may also be implicated in cancer formation. Karube et al [193] recently observed a diminution of Dicer expression in nonsmall cell lung cancer samples taken from 67 patients, as assessed by RT-PCR. As for the let-7 miRNA, this reduction was also associated with shorter postoperative survival [193]. Thus, the presence of Dicer, which mediates miRNA biogenesis, appears to be required for maintaining normal cell function.

miRNA expression profiling of the affected tissues may eventually be important for improving the diagnosis of diseases, such as cancer [194]. Using a new miRNA profiling method, Lu et al [195] analyzed mammalian miRNAs from 334 samples, including human cancers. They observed a characteristic general downregulation of miRNA expression in tumors, as compared to normal tissues [195]. Similarly, Jiang et al used an RT-PCR approach using primers specific to 222 pre-miRNAs to monitor their expression in human cancer cell lines [196]. Monitoring of global changes in miRNA expression profiles will be useful to establish possible links between miRNAs and diseases.

miRNAs and viruses

Several studies have reported a role for RNA silencing in host defense mechanisms against viruses in plants [197], and reports suggest that they may also play a similar role in humans. The interaction between RNA silencing pathways and viruses, such as HIV-1, is complex and multifaceted [198]. Some viral RNAs exhibit secondary structures that are prone to Dicer processing, as evidenced by the discovery of miRNAs derived from Epstein-Barr virus (EBV), a virus belonging to the herpesvirus family, in infected Burkitt's lymphoma cells [199]. In this case, miRNAs originate from five precursors present in two different clusters of the genome of EBV [199]. The overall impact of viral miRNAs on cellular and viral gene expression remains to be fully appreciated. They may target and regulate specific human mRNAs, thereby ultimately influencing cell function and viral replication. Indeed, the potential host mRNA targets of these miRNAs, as predicted by bioinformatical analyses, are implicated in many biological processes, such as transcription, cell proliferation, apoptosis, B cell-specific chemokine and cytokine synthesis, and signal transduction [199]. These findings illustrate how a virus may exploit the RNA silencing machinery for its own purpose.

miRNAs derived from a virus may also be turned against some of its mRNAs, as exemplified by miR-BART2. This EBV miRNA has been shown to be perfectly complementary to the EBV gene *BALF5*, encoding for a DNA polymerase, and to target it for degradation [199, 200].

The pathogenic human Kaposi's sarcoma-associated herpesvirus (KSHV) was recently shown to encode an array of 11 distinct miRNAs, all of which are expressed at readily detectable levels in latently KSHV infected cells [201]. Computer analysis of potential mRNA host targets for these viral miRNAs included several mRNAs previously shown to be downregulated in KSHV-infected cells, suggesting that KSHV miRNAs play a critical role in the establishment and/or maintenance of KSHV latent infection [201].

The genome of HIV-1 encodes a gene called *nef*, which is located in the 3′ region and is overlapping with the LTR. Omoto et al identified a *nef*-derived miRNA, called miR-N367, produced in cells persistently infected with HIV-1. This miRNA has been shown to downregulate the transcription of the HIV-1 genome in human T cells by targeting the negative responsive element of its 5′NTR U3 region and the *nef* sequence located in the 3′NTR [202, 203]. HIV-1 was also found to generate an siRNA that can mediate nucleic-acid-based immunity and to encode a suppressor of RNA silencing in its Tat protein [204].

Additional evidences suggest that viruses have evolved to take advantage of RNA silencing pathways to enhance the probability of successful infection. For example, the simian virus 40 (SV40) genome was found to encode a pre-miRNA from which two miRNAs can be derived. Expressed at late times in infection, these miRNAs are perfectly complementary to the early viral mRNAs, and target those for degradation [205]. SV40-infected cells show a reduced expression of viral T antigens, are less sensitive to lysis by cytotoxic T cells, and trigger less cytokine production [205].

Cellular miRNAs may also play an important role in virus/host interactions. For example, miR-32 was found to restrict retrovirus primate foamy virus type 1 (PFV-1) accumulation in human cells [166]. However, PFV-1 may counteract this cellular restriction through expression of Tas, a protein inhibiting RNA silencing in mammalian cells [166]. Moreover, a study showed that the sequestration of miR-122, an miRNA highly and specifically expressed in the liver,

resulted in a marked loss of autonomously replicating hepatitis C virus (HCV) RNAs [167]. HCV replication thus appears to be facilitated by a genetic interaction between miR-122 and the 5′NTR of the HCV genome, making miR-122 a potential target for an anti-HCV intervention.

As for a possible regulation of HIV-1 replication by human miRNAs, computational predictions identified four possible HIV-1 targets: the *nef* gene targeted by miR-29a and miR-29b, the *vpr* gene targeted by miR-149, the *vpu* gene targeted by miR-378, and the *vif* gene targeted by miR-324-5p [168]. Microarray profiling confirmed the expression of these miRNAs in HIV-1 replication-competent human T lymphocytes [168]. Further investigation is required to determine the biological significance of these cellular miRNAs-HIV-1 interactions.

CONCLUSION

miRNAs are now recognized as key regulators of gene expression. Not surprisingly, causal links between deregulation of miRNA expression and some important genetic diseases are gradually emerging. A better characterization of the miRNA expression profiles observed in various clinical situations may ultimately be useful to physicians in providing signatures for specific tumors or infectious diseases. Further investigations that aim at elucidating and understanding the mechanisms involved in miRNA biosynthesis and function are crucial for the design and development of potentially important diagnostic tools and new therapeutic strategies.

ACKNOWLEDGMENTS

We are grateful to Gilles Chabot for graphic design. M. P. Perron and L.-A. Gobeil are supported by doctoral and master studentships from the Natural Sciences and Engineering Research Council of Canada (NSERC), respectively. P. Provost is a New Investigator of the Canadian Institutes of Health Research (CIHR) and The Arthritis Society. This work was supported by Discovery Grant 262938-03 from NSERC and Grant EOP-64706 from Health Canada/CIHR. D. L. Ouellet and M. P. Perron contributed equally to this work. P. Provost is the author for correspondence.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 26818, Pages 1–13
DOI 10.1155/JBB/2006/26818

Review Article

Intronic MicroRNA (miRNA)

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Received 25 January 2006; Accepted 3 April 2006

Nearly 97% of the human genome is composed of noncoding DNA, which varies from one species to another. Changes in these sequences often manifest themselves in clinical and circumstantial malfunction. Numerous genes in these non-protein-coding regions encode microRNAs, which are responsible for RNA-mediated gene silencing through RNA interference (RNAi)-like pathways. MicroRNAs (miRNAs), small single-stranded regulatory RNAs capable of interfering with intracellular messenger RNAs (mRNAs) with complete or partial complementarity, are useful for the design of new therapies against cancer polymorphisms and viral mutations. Currently, many varieties of miRNA are widely reported in plants, animals, and even microbes. Intron-derived microRNA (Id-miRNA) is a new class of miRNA derived from the processing of gene introns. The intronic miRNA requires type-II RNA polymerases (Pol-II) and spliceosomal components for their biogenesis. Several kinds of Id-miRNA have been identified in *C elegans*, mouse, and human cells; however, neither function nor application has been reported. Here, we show for the first time that intron-derived miRNAs are able to induce RNA interference in not only human and mouse cells, but in also zebrafish, chicken embryos, and adult mice, demonstrating the evolutionary preservation of intron-mediated gene silencing via functional miRNA in cell and in vivo. These findings suggest an intracellular miRNA-mediated gene regulatory system, fine-tuning the degradation of protein-coding messenger RNAs.

INTRODUCTION

The first microRNA (miRNA) molecules, *lin-4* and *let-7*, were identified in 1993 [1]. Since then there have been rapid advances in small RNA research, with progress in identifying more miRNAs and understanding their biogenesis, functionality and target gene regulation. These early miRNAs were located in the noncoding regions between genes and transcribed by unidentified promoters; these are intergenic miRNAs. Most miRNAs studied at this stage were recognized as intergenic miRNA until 2003, when Ambros et al [1] discovered some tiny noncoding RNAs derived from the intron regions of gene transcripts.

In the meantime, Lin et al [1] demonstrated the biogenetic and gene silencing mechanisms of these intron-derived miRNAs, providing the first functional evidence for a new miRNA category: intronic miRNA. As shown in Table 1, several intronic miRNA molecules have been identified in *C elegans*, mouse, and human genomes [1–3] and some of their functions have been related to RNA interference (RNAi).

Introns occupy the largest proportion of noncoding sequences in the protein-coding DNA of a genome. The transcription of the genomic protein-coding DNA generates precursor messenger RNA (pre-mRNA), which contains four major parts including the 5′-untranslated region (UTR), the protein-coding exon, the noncoding intron, and the 3′-UTR. Broadly speaking, both the 5′- and 3′-UTR can be seen as a kind of intron extension; however, their processing during mRNA translation is different from the intron located between two protein-coding exons, termed the in-frame intron. The in-frame intron can range up to thirty or so kilobases and was initially thought to be a huge genetic waste in gene transcripts. Recently, this misconception was corrected by the observation of intronic miRNA. miRNA is usually about 18–25 oligonucleotides in length and is capable of either directly degrading its intracellular messenger RNA (mRNA) target or suppressing the protein translation of its targeted mRNA, depending on the complementarity between the miRNA and its target. In this way, the intronic miRNA is similar structurally and functionally to the previously described intergenic miRNAs, but differs from them in its unique requirement for Pol-II and RNA splicing components for biogenesis [2, 4, 5]. Approximately 10 ∼ 30% of a spliced intron is exported into the cytoplasm with a moderate half-life [6].

Table 1

miRNA	Species	Host gene (intron) (#)	Target gene(s)
miR-2a, -b2	Worm	Spi	
miR-7b	Mammal	Pituitary gland specific factor 1A (2) [NM174947]	Paired mesoderm homeobox protein 2b; HLHm5
miR-10b	Mammal	Homeobox protein HOX-4 (4)	
miR-11	*Drosophila*	E2F	
miR-13b2	*Drosophila*	CG7033	
miR-15b, -16-2	Mammal	Chromosome-associated polypeptide C	
miR-25, -93, -106b	Mammal	CDC47 homolog (13)	
miR-26a1, -26a2, -26b	Vertebrate	Nuclear LIM interactor-interacting factor 1, 2, 3	
miR-28	Human	LIM domain-containing preferred translocation parterner in lipoma [NM005578]	
miR-30c1, -30e	Mammal	Nuclear transcription factor Y subunit γ (5)	Transcription factor HES-1; PAI-1 mRNA-binding protein
miR-33	Vertebrate	Sterol regulatory element binding protein-2 (15)	RNA-dependent helicase p68; NAG14 protein
miR-101b	Human	RNA 3′-terminal phospate cyclase-like protein (8)	
miR-103, -107	Human	Pantothenate kinase 1, 2, 3	
miR-105-1, -105-2, -224	Mammal	γ-aminobutyric-acid receptor α-3 subunit precursor, epsilon subunit precursor	
miR-126, -126*	Mammal	EGF-like, Notch4-like, NEU1 protein (6) [NM178444]	
miR-128b	Mammal	cAMP-regulated phospho-protein 21 (11)	
miR-139	Mammal	cGMP-dependent 3′,5′-cyclic phosphodiesterase (2)	
miR-140	Human	NEDD4-like ubiquitin-protein ligase WWP2 (15)	
miR-148b	Mammal	Coatomer ζ-1 subunit	
miR-151	Mammal		
miR-152	Human	Coatomer ζ-2 subunit	N-myc proto-oncogene protein; noggin precursor
miR-153-1, -153-2	Human	Protein-tyrosine phosphatase N precursors	
miR-208	Mammal	Myosin heavy chain, cardiac muscle α isoform (28)	
miR-218-1, -218-2	Human	Slit homolog proteins [NM003062]	

RNA interference (RNAi) is a posttranscriptional gene silencing mechanism in eukaryotes, which can be triggered by small RNA molecules such as microRNA (miRNA) and small interfering RNA (siRNA). These small RNA molecules usually function as gene silencers, interfering with intracellular expression of genes either completely or partially complementary to the small RNAs. In principle, siRNAs are double-stranded RNAs capable of degrading target gene transcripts

with almost perfect complementarity [7, 8]. Unlike the stringent complementarity of siRNAs to their RNA targets, miRNAs are single-stranded and able to pair with target RNAs that have partial complementarity to the miRNAs [9, 10]. Numerous natural miRNAs are derived from hairpin-like RNA precursors in almost all eukaryotes, including yeast (*Schizosaccharomyces pombe*), plant (*Arabidopsis spp*), nematode (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*), mouse, and human, functioning as a defense against viral infections and allowing regulation of gene expression during development [11–21]. In contrast, natural siRNAs are abundant in plants and relatively simple animals (worms and flies), but are rarely seen in mammals [10]. Because of the widespread presence of miRNAs in eukaryotes, these small RNAs have recently been used to design novel therapeutics for cancers and viral infections [4, 22]. In fact, gene-silencing mechanisms involving miRNA may be an intracellular defense system for eliminating undesired transgenes and foreign RNAs, such as viral infections and retrotransposon activities [22, 23].

Definition of miRNA: Biogenesis

The definition of intronic miRNAs is based on two factors; first, they must share the same promoter with their encoded target genes, and second, they are spliced out of the transcript of such encoded genes and further processed into mature miRNAs. Although some of the currently known miRNAs are encoded in the genomic intron region of a given gene but in the opposite orientation to the gene transcript (palindrome), such miRNAs are not intronic miRNAs because they neither share the same promoter with the gene nor need to be released from the gene transcript by RNA splicing. For the transcription of such palindromic miRNAs, the promoters are located in the antisense direction to the gene, likely using the gene transcript as a potential target for the antisense miRNA. A good example is *let-7c*, which is an intergenic miRNA located in the antisense region of a gene intron. Current computer programs for miRNA prediction cannot distinguish the intronic miRNA from the intergenic miRNA. Because intronic miRNAs are encoded in the gene transcript precursors (pre-mRNA) and share the same promoter with the encoded gene transcripts, the miRNA prediction programs tend to classify the intronic miRNAs along with the intergenic miRNAs located in the exonic regions. However, in view of their different biogenic mechanisms, these two types of miRNA may have different gene-regulatory functions in the adjustment of cellular physiology. Thus, a miRNA-prediction program utilizing a database of noncoding sequences located in the protein-coding pre-mRNA regions is urgently needed for thoroughly screening and understanding the distribution and variety of hairpin-like intronic miRNAs in the genomes.

The process of miRNA biogenesis in vertebrates involves five steps (Figure 1). First, a long primary precursor miRNA (pri-miRNA) is excised, probably by RNA polymerase type-II (Pol-II) [2, 24]. Second, the long pri-miRNA is further excised by Drosha-like RNase III endonucleases or spliceosomal components, depending on the origin of the pri-miRNA either in an exon or an intron, respectively [2, 25], to form a mature precursor miRNA (pre-miRNA), and third, the pre-miRNA is exported out of the nucleus by Ran-GTP and the receptor Exportin-5 [26, 27]. In the cytoplasm, Dicer-like nucleases cleave the pre-miRNA to form mature miRNA. Lastly, the mature miRNA is incorporated into a ribonuclear particle (RNP), which becomes the RNA-induced gene silencing complex (RISC), capable of executing RNAi-related gene silencing [28, 29]. Although an in vitro model of siRNA-associated RISC assembly has been generated, the link between the final miRNA maturation and RISC assembly remains to be determined. The characteristics of Dicer and RISC are distinctly different in the siRNA and miRNA mechanisms [30]. In zebrafish, we have recently observed that the stem-loop structure of pre-miRNA is involved in strand selection for mature miRNA during RISC assembly. These findings suggest that the duplex structure of siRNA may not be essential for the assembly of miRNA-associated RISC in vivo. The biogeneses of miRNA and siRNA seem to be very similar; however, the miRNA mechanisms previously proposed were based on the model of siRNA. In contrast, it will be necessary to distinguish the individual properties and differences in these two types of RNAs in order to understand the evolutionary and functional relationship of these gene-silencing pathways. In addition, the differences may provide a clue for understanding the prevalence of native siRNAs in invertebrates compared to that in mammals.

The proposed research will generate data from several transgenic zebrafish lines. It is our explicit intention that these data will be submitted in a readily accessible public database in the ZFIN website. All efforts will be made to rapidly release data through publication of results as quickly as possible to analyze the experiments. Data used in publications will be released in a timely manner. ZFIN data will be made accessible through a public site that allows querying as has been set up for a similar project.

Intronic miRNA and disease

The majority of human gene transcripts contain introns, phylogenetically conserved to a greater or lesser degree. Changes in these non-protein-coding sequences are frequently observed in clinical malfunction such as myotonic dystrophy and fragile X syndrome.

Numerous introns encode miRNAs which are involved in RNAi-related chromatin silencing mechanisms. Over 90 intronic miRNAs have been identified using the bioinformatic approaches to date, but the function of the vast majority of these molecules remains to be determined [3]. According to the strictly expressive correlation of intronic miRNAs with their encoded genes, one may speculate that the levels of condition-specific, time-specific, and individual-specific gene expression are determined by the influences of distinctive miRNAs on single or multiple gene modulation. This interpretation accounts for the heterogeneity of

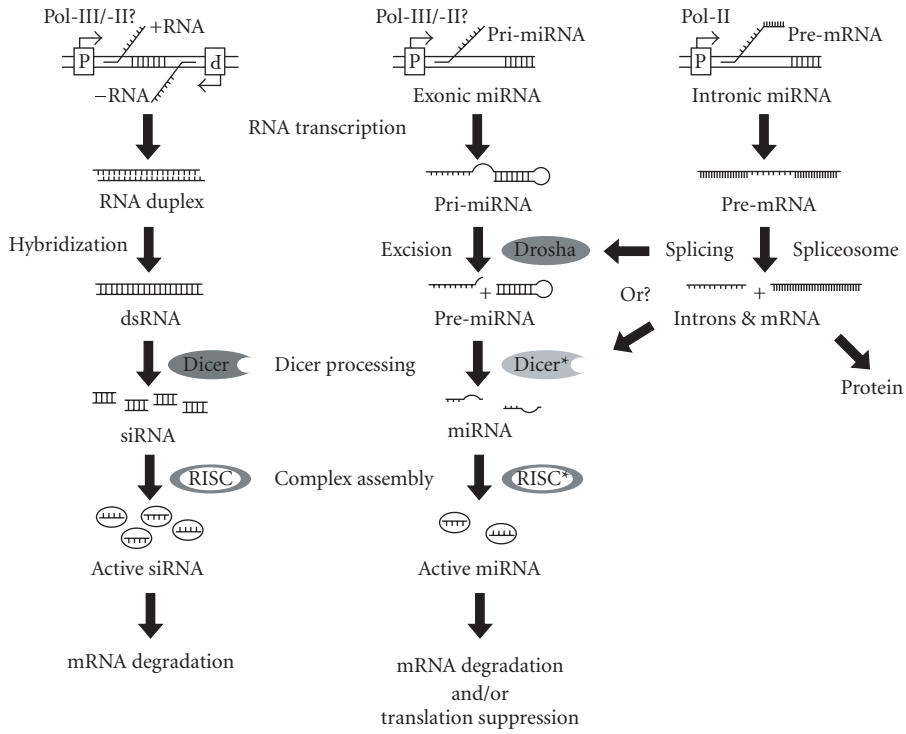

Figure 1: Comparison of biogenesis and RNAi mechanisms among siRNA, intergenic (exonic) miRNA, and intronic miRNA. siRNA is likely formed by two perfectly complementary RNAs transcribed from two different promoters (remaining to be determined) and further processing into 19–22 bp duplexes by the RNase III-familial endonuclease, Dicer. The biogenesis of intergenic miRNAs, for example, *lin-4* and *let-7*, involves a long transcript precursor (pri-miRNA), which is probably generated by Pol-II or Pol-III RNA promoters, while intronic miRNAs are transcribed by the Pol-II promoters of its encoded genes and coexpressed in the intron regions of the gene transcripts (pre-mRNA). After RNA splicing and further processing, the spliced intron may function as a pri-miRNA for intronic miRNA generation. In the nucleus, the pri-miRNA is excised by Drosha RNase to form a hairpin-like pre-miRNA template and then exported to the cytoplasm for further processing by Dicer* to form mature miRNA. The Dicers for siRNA and miRNA pathways are different. All three small regulatory RNAs are finally incorporated into an RNA-induced silencing complex (RISC), which contains either the strand of siRNA or the single-strand of miRNA. The action of miRNA is considered to be more specific and less adverse than that of siRNA because only one strand is involved. siRNA primarily triggers mRNA degradation, whereas miRNA can induce either mRNA degradation or suppression of protein synthesis depending on the sequence complementarity to the target gene transcripts.

genetic expression of various traits; dysregulation will result in genetic disease. For instance, monozygotic twins frequently demonstrate slight, but definitely distinguishing, differences in disease susceptibility and behavior. For example, a long CCTG expansion in intron 1 of the zinc finger protein ZNF9 gene has been correlated with type 2 myotonic dystrophy in whichever twin exhibits the higher susceptibility [31]. Since the expansion motif bound with high affinity to certain RNA-binding proteins, an interfering role of intron-derived expansion fragments is suggested. Another more-established example involving pathogenic intronic expansion fragments is fragile X syndrome, which accounts for about 30% of human inherited mental retardation. Intronic CGG repeat (rCGG) expansion in the 5′-UTR of the *FMR1* gene is the causative mutation in 99% of individuals with fragile X syndrome [32]. *FMR1* encodes an RNA-binding protein, FMRP, which is associated with polyribosome assembly in an RNP-dependent manner and is capable of suppressing translation through an RNAi-like pathway. FMRP also contains a nuclear localization signal (NLS) and a nuclear export signal (NES) for shuttling certain mRNAs between the nucleus and cytoplasm [33]. Jin et al proposed that RNAi-mediated methylation occurs in the CpG region of the *FMR1* rCGG expansion, which is targeted by a hairpin RNA derived from the 3′-UTR of the *FMR1* expanded allele transcript [32]. The Dicer-processed hairpin RNA triggers the formation of an RNA-induced initiator of transcriptional gene silencing (RITS) on the homologous rCGG sequences and leads to heterochromatin repression of the *FMR1* locus. These examples suggest that natural evolution gives rise to more intronic complexity and variety in higher animals and plants, allowing the coordination of their vast gene expression libraries and interactions. Any dysregulation of miRNA derivation from introns may then lead to genetic disease involving intronic expansion or deletion, such as myotonic dystrophy and fragile X mental retardation.

Man-made intronic miRNA

To understand the disease caused by the dysregulation of intronic miRNA, an artificial expression system is needed to recreate the function and mechanism of miRNA in vitro and in vivo. The same approach may be used to design and develop therapies. Several vector-based RNAi expression systems have been developed, using type-III RNA polymerase (Pol-III)-directed transcription activities, to generate more stable RNAi efficacy and lower interferon-related toxicity in several cell lines in vitro [34–37]. For gene therapy in vivo, a functional gene is preferably delivered into an animal or human being by expression-competent vector vehicles, such as retroviral vector, lentiviral vector, adenoviral vector, and adenoassociated viral (AAV) vector. The main purpose of these vector-based approaches is to maintain long-term and consistent gene modulation. Although some studies [38, 39] attempting to use the Pol-III-directed RNAi system have succeeded in maintaining constant gene silencing efficacy in vivo, their delivery strategies failed to target a specific cell population due to the ubiquitous existence of Pol-III activity in all cell types. Moreover, the requirement of using Pol-III RNA promoters, for example, U6 and H1, for small RNA expression is another problem. Because the read-through side-effect of Pol-III occurs on a short transcription template in the absence of proper termination, large RNA products longer than the desired 18–25 base pairs (bp) can be synthesized and cause unexpected interferon cytotoxicity [40, 41]. Such a problem can also result from competition between the Pol-III promoter and another vector promoter (ie, LTR and CMV promoters). We and others [42] have found that a high dosage of siRNA (eg, > 250 nM in human T cells) caused strong cytotoxicity similar to that of long double-stranded dsRNA [42, 43]. This toxicity is due to the double-stranded structure of siRNA and dsRNA, which activates interferon-mediated nonspecific RNA degradation and programmed cell death through signaling via the PKR and 2–5A systems. It is well known that the interferon-induced protein kinase PKR can trigger cell apoptosis, while activation of the interferon-induced 2′, 5′-oligoadenylate synthetase (2–5A) system leads to extensive cleavage of single-stranded RNAs (ie, mRNAs) [44]. Both the PKR and 2–5A systems contain dsRNA-binding motifs which are highly conserved, but these motifs do not bind either single-strand RNAs or RNA-DNA hybrids. These disadvantages limit the use of Pol-III-based RNAi vector systems for therapeutic purposes.

The intron-derived miRNA system is activated in a specific cell type under the control of type-II RNA polymerases (Pol-II)-directed transcriptional machinery. To overcome Pol-III-mediated siRNA side effects, we have successfully developed a novel Pol-II-based miRNA biogenesis strategy, employing intronic miRNA molecules [2] to knock down more than 85% of selected oncogene function or viral genome replication [45, 46]. Because of the flexibility in binding to partially complementary mRNA targets, miRNA can serve as an anticancer drug or vaccine, a major breakthrough in the treatment of cancer polymorphisms and viral mutations. We are the first research group to discover the biogenesis of miRNA-like precursors from the 5′-proximal intron regions of gene transcripts (pre-mRNA) produced by the mammalian Pol-II. Depending on the promoter of the miRNA-encoded gene transcript, intronic miRNA is coexpressed with its encoding gene in a specific cell population, which activates the promoter and expresses the gene. It has been noted that a spliced intron was not completely digested into monoribonucleotides for transcriptional recycling since approximately 10–30% of the intron was found in the cytoplasm with a moderate half life [6, 47]. This type of miRNA generation relies on the coupled interaction of nascent Pol-II-mediated pre-mRNA transcription and intron excision, occurring within certain nuclear regions proximal to genomic perichromatin fibrils [46, 48, 49]. After Pol-II RNA processing and splicing excision, some of the intron-derived miRNA fragments can form mature miRNAs and effectively silence the target genes through the RNAi mechanism, while the exons of pre-mRNA are ligated together to form a mature mRNA for protein synthesis (Figure 2(a)) [2]. Because miRNAs are single-stranded molecules insensitive to PKR- and 2–5A-induced interferon systems, the Pol-II-mediated miRNA generation can avoid the cytotoxic effects of dsRNA and siRNA in vitro and in vivo. These findings indicate new functions for mammalian introns in intracellular miRNA generation and gene silencing, which can be used both as tools for the analysis of gene functions and the development of gene-specific therapeutics against cancers and viral infections.

Using artificial introns carrying hairpin-like miRNA precursors (pre-miRNA), we have successfully generated mature miRNA molecules with full capacity for triggering RNAi-like gene silencing in human prostate cancer LNCaP, human cervical cancer HeLa, and rat neuronal stem HCN-A94-2 cells [2, 45]. As shown in Figure 2(b), the artificial intron (SpRNAi) was cotranscribed within a precursor messenger RNA (pre-mRNA) by Pol-II and cleaved out of the pre-mRNA by RNA splicing. Then the spliced intron containing the pre-miRNA was further processed into mature miRNA capable of triggering RNAi-related gene-silencing effects. Utilizing this artificial miRNA model, we have tested various pre-miRNA constructs, and observed that the production of intron-derived miRNA fragments originated from the 5′-proximity of the intron sequence between the 5′-splice site and the branching point. These miRNAs were able to trigger strong suppression of genes possessing more than 70% complementarity to the miRNA sequences, whereas nonhomologous miRNA intron, that is, empty intron without the pre-miRNA insert, with an off-target miRNA insert (negative control) and splicing-defective intron, showed no silencing effects on the targeted gene. The same results can also be reproduced in the zebrafish by directing the miRNA against target EGFP expression (Figure 2(c)), indicating the consistent preservation of the intronic miRNA biogenesis system in vertebrates. Furthermore, no effect was detected on off-target genes, such as RGFP and β-actin, suggesting the high specificity of miRNA-directed RNA interference (RNAi). We have confirmed the identity of the intron-derived miRNA, which comprised about 18–25 nucleotides

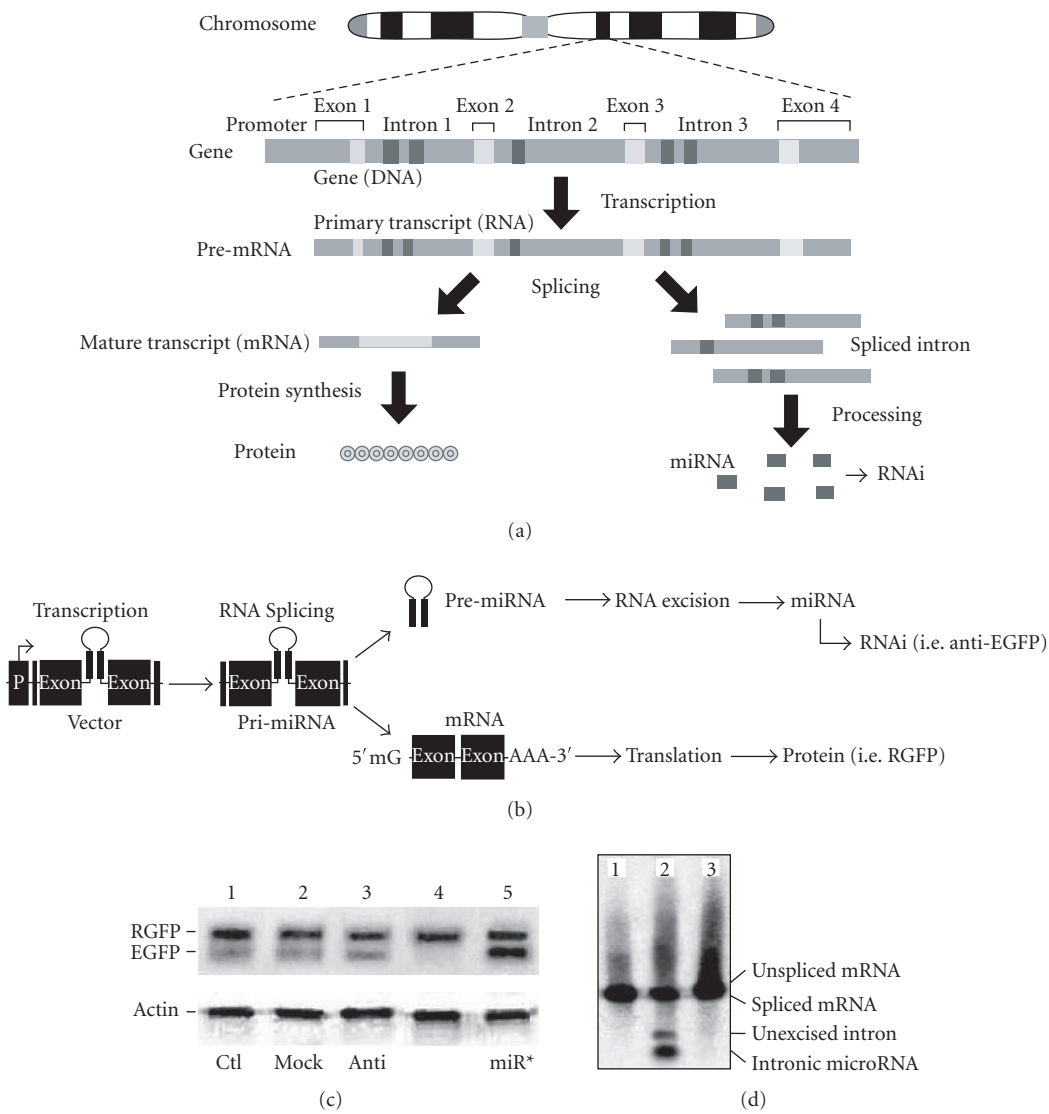

Figure 2: Biogenesis and function of intronic miRNA. (a) The native intronic miRNA is cotranscribed with a precursor messenger RNA (pre-mRNA) by Pol-II and cleaved out of the pre-mRNA by an RNA splicing machinery, the spliceosome. The spliced intron with hairpin-like secondary structure is further processed into mature miRNA capable of triggering RNAi effects, while the ligated exons become a mature messenger RNA (mRNA) for protein synthesis. (b) We designed an artificial intron containing pre-miRNA, namely SpRNAi, mimicking the biogenesis of the native intronic miRNA. (c) When a designed miR-EGFP(280–302)-stemloop RNA construct was tested in the EGFP-expressing Tg(UAS:gfp) zebrafish, we detected a strong RNAi effect only on the target EGFP (lane 4). No detectable gene-silencing effect was observed in other lanes; from left to right: 1, blank vector control (Ctl); 2, miRNA-stemloop targeting HIV-p24 (mock); 3, miRNA without stemloop (anti); and 5, stemloop-miRNA* complementary to the miR-EGFP(280–302) sequence (miR*). The off-target genes, such as vector RGFP and fish actin, were not affected, indicating the high target specificity of miRNA-mediated gene silencing. (c) Three different miR-EGFP(280–302) expression systems were tested for miRNA biogenesis; from left to right: 1, vector expressing intron-free RGFP, no pre-miRNA insert; 2, vector expressing RGFP with an intronic 5′-miRNA-stemloop-miRNA*-3′ insert; and 3, vector similar to the 2 construct but with a defected 5′-splice site in the intron. In Northern blot analysis probing the miR-EGFP(280–302) sequence, the mature miRNA was released only from the spliced intron resulting from the vector 2 construct in the cell cytoplasm.

(nt), approximately the length of the newly identified intronic miRNAs in *C elegans*. Moreover, the intronic small RNAs isolated by guanidinium-chloride ultracentrifugation can elicit strong, but short-lived, gene-silencing effects on the homologous genes in transfected cells, indicating a reversible RNAi effect. Thus, the long-term (> 1 month) gene-silencing effect that we observed in vivo, using the Pol-II-mediated intronic miRNA system, is likely maintained by constitutive miRNA production from the vector rather than the stability of the miRNA.

The components of the Pol-II-mediated SpRNAi system include several consensus nucleotide elements consisting of

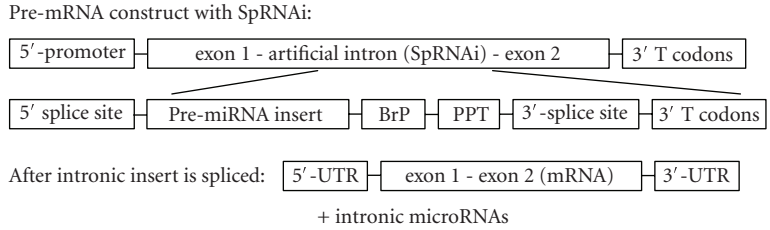

Figure 3: Schematic construct of the artificial SpRNAi intron in a recombinant gene SpRNAi-RGFP for intracellular expression and processing. The components of the Pol-II-mediated SpRNAi system include several consensus nucleotide elements consisting of a 5′-splice site, a branch-point domain (BrP), a poly-pyrimidine tract (PPT), a 3′-splice site, and a pre-miRNA insert located between the 5′-splice site and the BrP domain. The expression of the recombinant gene is under the regulation of either a mammalian Pol-II RNA promoter or a compatible viral promoter for cell-type-specific effectiveness. Mature miRNA molecules are released from the intron by RNA splicing and further Dicer processing.

a 5′-splice site, a branch-point domain, a poly-pyrimidine tract, and a 3′-splice site (Figure 3). Additionally, a pre-miRNA insert sequence is placed within the artificial intron between the 5′-splice site and the branch-point domain. This portion of the intron would normally form a lariat structure during RNA splicing and processing. We now know that spliceosomal U2 and U6 snRNPs, both helicases, may be involved in the unwinding and excision of the lariat RNA fragment into pre-miRNA; however, the detailed processing remains to be elucidated. Further, the SpRNAi contains a translation stop codon domain (T codon) in its 3′-proximal region to facilitate the accuracy of RNA splicing which, if present in a cytoplasmic mRNA, would signal the diversion of a splicing-defective pre-mRNA to the nonsense-mediated decay (NMD) pathway and thus cause the elimination of any unspliced pre-mRNA in the cell. For intracellular expression of the SpRNAi, we needed to insert the SpRNAi construct into the *Dra*II cleavage site of a red fluorescent membrane protein (RGFP) gene from mutated chromoproteins of coral reef *Heteractis crispa*. The cleavage of RGFP at its 208th nucleotide site by the restriction enzyme *Dra*II generates an AGGN nucleotide break with three recessing nucleotides at each end, which forms 5′ and 3′ splice sites, respectively, after the SpRNAi insertion. Because this intronic insertion disrupts the expression of functional RGFP, it becomes possible to determine the occurrence of intron splicing and RGFP-mRNA maturation through the appearance of red fluorescent emission around the membrane surface of the transfected cells. The RGFP also provides multiple exonic splicing enhancers (ESEs) to increase RNA splicing efficiency.

Intron-mediated gene silencing in zebrafish

The foregoing discussion establishes the fact that intronic miRNAs are an effective strategy for silencing specific target genes in vivo. We first tried to determine the structural design of pre-miRNA inserts for the best gene-silencing effect. We found that a strong structural bias exists for the selection of a mature miRNA strand during the assembly of the RNAi effector, the RNA-induced gene silencing complex (RISC). RISC is a protein: RNA complex that directs either target gene transcript degradation or translational repression through the RNAi mechanism. Formation of siRNA duplexes plays a key role in the assembly of the siRNA-associated RISC. The two strands of the siRNA duplex are functionally asymmetric, but the assembly into the RISC complex is preferential for only one strand. Such preference is determined by the thermodynamic stability of each 5′-end base-pairing in the strand. Based on this siRNA model, the formation of miRNA and its complementary miRNA (miRNA*) duplex was thought to be an essential step for the assembly of miRNA-associated RISC. If this were true, no functional bias would be observed in the stemloop of a pre-miRNA. Nevertheless, we observed that the stemloop of the intronic pre-miRNA was involved in the strand selection of a mature miRNA for RISC assembly in zebrafish. In these experiments, we constructed miRNA-expressing SpRNAi-RGFP vectors as previously described [2] and two symmetric pre-miRNAs, miRNA-stemloop-miRNA* (1) and miRNA*-stemloop-miRNA (2), were synthesized and inserted into the vectors, respectively. Both pre-miRNAs contained the same double-stranded stem arm region, which was directed against the EGFP nt 280–302 sequence. Because the intronic insert region of the SpRNAi-RGFP recombined gene is flanked with a *PvuI* and an *MluI* restriction site at the 5′- and 3′-ends, respectively, the primary insert can be easily removed and replaced by various gene-specific inserts (eg, anti-EGFP) possessing cohesive ends. By allowing a change in the pre-miRNA inserts directed against different gene transcripts, this intronic miRNA generation system provides a valuable tool for genetic and miRNA-associated research in vivo.

To determine the structural preference of the designed pre-miRNA, we have isolated the zebrafish small RNAs by mirVana miRNA isolation columns (Ambion, Austin, TX) and then precipitated all potential miRNAs complementary to the target EGFP region by latex beads containing the target RNA sequence. One full-length miRNA, miR-EGFP(280–302), was active in the transfections of the 5′-miRNA-stemloop-miRNA*-3′ construct, as shown in Figure 4(a) (gray-shading sequences). Since the mature miRNA was detected only in the zebrafish transfected by the 5′-miRNA-stemloop-miRNA*-3′ construct, the miRNA-associated RISC tends to preferably interact

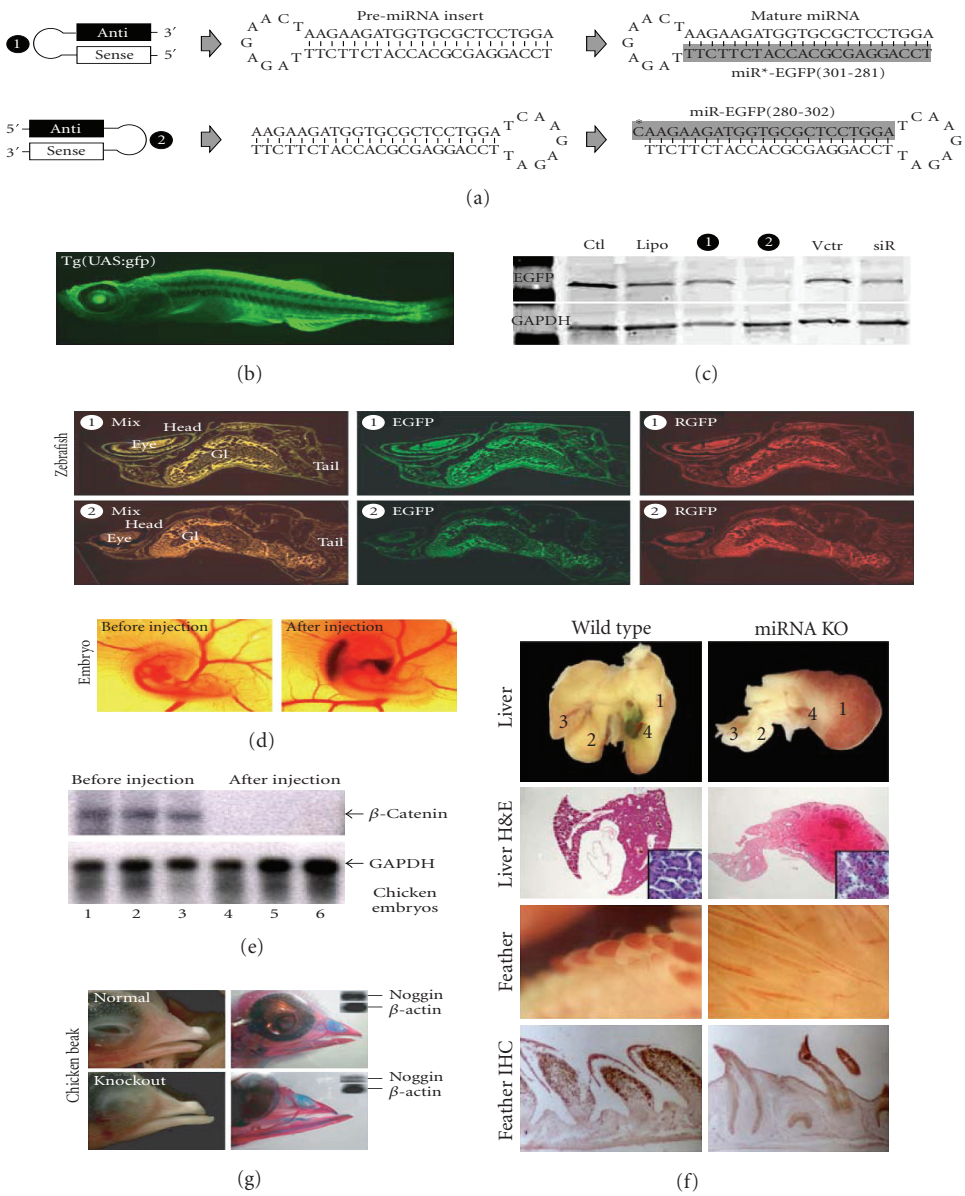

Figure 4: Intronic miRNA-mediated gene silencing effects in vivo. (a)–(c) Different preferences for RISC assembly were observed following the transfection of 5′-miRNA*-stemloop-miRNA-3′ (1) and 5′-miRNA-stemloop-miRNA*-3′ (2) pre-miRNA structures in zebrafish, respectively. (a) One mature miRNA, namely miR-EGFP(280/302), was detected in the (2)-transfected zebrafish, whereas the (1)-transfection produced another kind of miRNA, miR*-EGFP(301–281), which was partially complementary to the miR-EGFP(280/302). (b) The RNAi effect was only observed with the transfection of the (2) pre-miRNA, showing less EGFP (green) in (2) than in (1), while the miRNA indicator RGFP (red) was equally present in all vector transfections. (c) Western blot analysis of the EGFP protein levels confirmed the specific silencing result of (b). No detectable gene silencing was observed in fish without (Ctl) and with liposome only (Lipo) treatments. The transfection of either a U6-driven siRNA vector (siR) or an empty vector (Vctr) without the designed pre-miRNA insert resulted in no significant gene silencing. (d)–(g) Silencing of endogenous β-catenin and noggin genes in chicken embryos. (d) The pre-miRNA construct and fast green dye mixtures were injected into the ventral side of chicken embryos near the liver primordia below the heart. (e) Northern blot analysis of extracted RNAs from chicken embryonic livers with anti-β-catenin miRNA transfections (lanes 4–6) in comparison with wild types (lanes 1–3) showed a more than 98% silencing effect on β-catenin mRNA expression, while the house-keeping gene, GAPDH, was not affected. (f) Liver formation of the β-catenin knockouts was significantly hindered (upper right 2 panels). Microscopic examination revealed a loose structure of hepatocytes, indicating the loss of cell-cell adhesion due to breaks in adherents junctions formed between β-catenin and cell membrane E-cadherin in early liver development. In severely affected regions, feather growth in the skin close to the injection area was also inhibited (lower right 2 panels). Immunohistochemical staining for β-catenin protein (brown) showed a significant decrease in the feather follicle sheaths. (g) Lower beak development was increased by the mandibular injection of the antinoggin pre-miRNA construct (lowerpanel) in comparison to wild type (upper panel). Right panels showed bone (alizarin red) and cartilage (alcian blue) staining to demonstrate the outgrowth of bone tissues in the lower beak of the noggin knockout. Northern blot analysis (small windows) confirmed a $\sim$ 60% decrease of noggin mRNA expression in the lower beak area.

with the construct (2) rather than the (1) pre-miRNA. The green fluorescent protein EGFP expression was constitutively driven by the β-actin promoter located in almost all cell types of the zebrafish, while Figure 4(b) shows that transfection of the SpRNAi-RGFP vector into the Tg(UAS:gfp) zebrafish coexpressed the red fluorescent protein RGFP, serving as a positive indicator for miRNA generation in the transfected cells. This approach has been successfully used in several mouse and human cell lines to show RNAi effects [2, 45]. We applied the liposome-encapsulated vector (total 60 μg) to the fish and found that the vector easily penetrated almost all tissues of the two-week-old zebrafish larvae within 24 hours, providing fully systemic delivery of the miRNA effect. The indicator RGFP was detected in both of the fish transfected by either 5′-miRNA*-stemloop-miRNA-3′ or 5′-miRNA-stemloop-miRNA*-3′ pre-miRNA, whereas the silencing of target EGFP expression (green) was observed only in the fish transfected by the 5′-miRNA-stemloop-miRNA*-3′ pre-miRNA (Figures 4(b)–4(c)). The suppression level in the gastrointestinal (GI) tract was somewhat lower, probably due to the high RNase activity in this region. Because thermostability in the 5′ end of the siRNA duplexes resulting from both of the designed pre-miRNA molecules is the same, we suggest that the stemloop of pre-miRNA is involved in strand selection of mature miRNA during RISC assembly. Given that the cleavage site of Dicer in the stem arm determines the strand selection of mature miRNA [25], the stemloop may function as a determinant for the recognition of a special cleavage site. Therefore, the heterogeneity of stemloop structures among various species may help to explain the prevalence of native miRNA in vertebrates over invertebrates.

Intron-mediated gene silencing in chicken embryos

The in vivo model of chicken embryos has been widely utilized in developmental biology, signal transduction, and flu vaccine development. We have successfully demonstrated the feasibility of localized gene silencing in vivo by the intronic miRNA approach and also discovered that the interaction between pre-mRNA and genomic DNA may be essential for miRNA biogenesis. As an example, the β-catenin gene was selected because its products play a critical role in development [50]. β-catenin is involved in the growth control of skin and liver tissues in chicken embryos. The loss-of-function of β-catenin is lethal in transgenic animals. As shown in Figures 4(d)–4(g), experimental results demonstrated that the miRNAs derived from a long mRNA-DNA hybrid construct ($\geq$ 150 bp) were capable of inhibiting β-catenin gene expression in the liver and skin of developing chicken embryos. Homologous recombination between the intronic miRNA and genomic DNA may account for a part of the specific gene-silencing effect [46]. We have demonstrated that the [P^{32}]-labeled DNA component of an mRNA-DNA duplex construct in cell nuclear lysates was intact during the effective period of miRNA-induced RNA interference (RNAi) phenomena, while the labeled RNA component was replaced by cold homologues and excised into small RNA fragments within a 3-day incubation period. Since intronic miRNA generation relies on a coupled interaction of nascent Pol-II-directed pre-mRNA transcription and intron excision occurring proximal to genomic perichromatin fibrils, the above observation indicates that pre-mRNA-genomic DNA recombination may facilitate new miRNA generation by Pol-II RNA transcription and excision for relatively long-term gene silencing. Alternatively, Pol-II may function as an RNA-dependent RNA polymerase (RdRp) for producing small interfering RNAs, since mammalian Pol-II possesses RdRp activities [51, 52]. Thus, it appears that Pol-II-mediated RNA generation and excision is involved in both mRNA-DNA-derived and intron-derived miRNA biogenesis, resulting in single-stranded small RNAs of about 20 nt, comparable to the usual sizes of Dicer-processed miRNAs as observed in the regulation of numerous developmental events.

In an effort to test the pre-mRNA and genome interaction theory, we performed an intracellular transfection of the mRNA-DNA hybrid construct containing a hairpin anti-β-catenin pre-miRNA, which was directed against the central region of the β-catenin coding sequence (aa 306–644) with perfect complementarity. A perfectly complementary miRNA theoretically directs target mRNA degradation more efficiently than translational repression. Using embryonic day 3 chicken embryos, a dose of 25 nM of the pre-miRNA construct was injected into the ventral body cavity, which is close to where the liver primordia would form (Figure 4(d)). For efficient delivery into target tissues, the pre-miRNA construct was mixed with the DOTAP liposomal transfection reagent (Roche Biomedicals, Indianapolis, IN) at a ratio of 3:2. A 10% (v/v) fast green solution was concurrently added during the injection as a dye indicator. The mixtures were injected into the ventral side near the liver primordia below the heart using heat pulled capillary needles. After injection, the embryonic eggs were sealed with sterilized scotch tape and incubated in a humidified incubator at 39–40° C until day 12 when the embryos were examined and photographed under a dissection microscope. Several malformations were observed, although the embryos survived without visible overt toxicity or overall perturbation of embryo development. The liver was the closest organ to the injection site and its phenotype was most dramatically affected. Other regions, particularly the skin close to the injection site, were also affected by the diffused miRNA. As shown in Figure 4(e), Northern blot analysis for the targeted β-catenin mRNA expression in the dissected livers showed that β-catenin expression in the wild-type livers remained normal (lanes 1–3), whereas expression in the miRNA-treated samples was decreased dramatically (lanes 4–6). miRNA silencing degraded more than 98% of β-catenin mRNA expression in the embryonic chicken, but had no effect on the house-keeping gene GAPDH expression, indicating high target specificity and very limited interferon-related cytotoxicity in vivo for the miRNA construct.

After ten days of primordial injection with the anti-β-catenin pre-miRNA template, the embryonic chicken livers showed enlarged and engorged first lobes, but the sizes of the second and third lobes of the livers were dramatically

decreased (Figure 4(f)). Histological sections of normal livers showed hepatic cords and sinusoidal space with few blood cells. In the anti-β-catenin miRNA-treated embryos, the general architecture of the hepatic cells in lobes 2 and 3 remained unchanged; however, there were islands of abnormality in lobe 1. Endothelial development appeared to be defective and blood leaked from the blood vessels. Abnormal hematopoietic cells were also observed between hepatocytes, particularly dominated by a population of small cells with round nuclei and scanty cytoplasm. In severely affected regions, hepatocytes were disrupted (Figure 4(f), small windows) and the diffused miRNA also inhibited feather growth in the skin area close to the injection site. The results showed that the anti-β-catenin miRNA was very effective in knocking out targeted gene expression at a very low dose of 25 nM over a long period of time ($\geq$ 10 days). Further, the miRNA gene-silencing effect appeared to be very specific as off-target organs appeared normal, indicating that the small single-stranded miRNA herein produced no generalized toxicity. In an attempt to silence noggin expression in the mandible beak area using the same approach (Figure 4(g)), an enlarged lower beak morphology was produced similar to what is seen in BMP4-overexpressing chicken embryos [53, 54]. Skeleton staining showed outgrowth of bone and cartilage tissues in the injected mandible area (Figure 4(g), right panels) and Northern blot analysis further confirmed that about 60% of noggin mRNA expression was knocked out in this region (small windows). Since bone morphogenetic protein 4 (BMP4), a member of the transforming growth factor-β (TGF-β) superfamily, is known to promote bone development and since noggin is an antagonist of BMP2/4/7 genes, it is not surprising to find that our miRNA-mediated noggin knockouts exhibited a morphological change resembling the effects of BMP4-overexpression as reported in chicken and other avian models. Thus, gene silencing in the chicken by miRNA transfection has a great potential for localized transgene-like manipulation in developmental biology.

Development of miRNA therapy

The following experiments demonstrate silencing exogenous retrovirus replication in an ex vivo cell model of patient-extracted $CD4^+$ T lymphocytes. Specific anti-HIV SpRNAi-RGFP vectors were designed to target the *gag-pol* region from approximately nt +2113 to +2450 of the HIV-1 genome. This region is relatively conserved and can serve as a good target for anti-HIV treatment [55]. The viral genes located in this target region include 3′-proximal $Pr55^{gag}$ polyprotein (ie, matrix p17 + capsid p24 + nucleocapsid p7) and 5′-proximal $p66/p51^{pol}$ polyprotein (ie, protease p10 + reverse transcriptase); all these components have critical roles in viral replication and infectivity. During the early infection phase, the viral reverse transcriptase transcribes the HIV RNA genome into a double-stranded cDNA sequence, which forms a preintegration complex with the matrix, integrase, and viral protein R (Vpr). This complex is then transferred to the cell nucleus and integrated into the host chromosome, consequently establishing the HIV provirus. We hypothesized that, although HIV carries few reverse transcriptase and matrix proteins during its first entry into host cells, the co-suppression of $Pr55^{gag}$ and $p66/p51^{pol}$ gene expression by miRNAs would eliminate the production of infectious viral particles in the late infection phase. Silencing $Pr55^{gag}$ may prevent the assembly of intact viral particles due to the lack of matrix and capsid proteins, while suppression of protease in $p66/p51^{pol}$ can inhibit the maturation of several viral proteins. HIV expresses about nine viral gene transcripts which encode at least 15 various proteins; thus, the separation of a polyprotein into individual functional proteins requires the viral protease activity. As shown in Figure 5, this therapeutic approach is feasible [22, 43].

The anti-HIV SpRNAi-RGFP vectors were tested in $CD4^+$ T lymphocyte cells from HAART-treated, HIV-seropositive patients. Because only partial complementarity between miRNA and its target RNA is needed to trigger the gene silencing effect, this approach may be superior to current small molecule drugs since the high rate of HIV mutations often produce resistance to such agents. Northern blot analysis in Figure 5(a) demonstrated the ex vivo gene silencing effect of anti-HIV miRNA transfection ($n = 3$ for each set) on HIV-1 replication in $CD4^+$ T lymphocytes from both acute and chronic phase AIDS patients. In the acute phase ($\leq$ one month), the 50 nM miRNA vector transfection degraded an average of 99.8% of the viral RNA genome (lane 4), whereas the same treatment knocked down only an average of $71.4 \pm 12.8\%$ of viral genome replication in the chronic phase (about a 2-year infection). Immunocytochemical staining for HIV p24 marker protein confirmed the results of Northern blot analysis (Figure 5(b)). Sequencing analysis has revealed at least two HIV-1b mutations in the acute phase and seven HIV-1b mutations in the chronic phase within the targeted HIV genome domain. It is likely that the higher genome complexity produced by HIV mutations in chronic infections reduces miRNA-mediated silencing efficacy. Transfection of 50 nM miRNA* vector homologous to the HIV-1 genome failed to induce any RNAi effect on the viral genome, indicating the specificity of the miRNA effect (lane 5). Expression of the cellular house-keeping gene, β-actin, was normal and showed no interferon-induced nonspecific RNA degradation. These results suggest that the designed anti-HIV SpRNAi-RGFP vector is highly specific and efficient in suppressing HIV-1 replication in early infections. In conjunction with an intermittent interleukin-2 therapy [55], the growth of noninfected $CD4^+$ T lymphocytes may be stimulated to eliminate the HIV-infected cells.

CONCLUSION

The consistent evidence of miRNA-induced gene silencing effects in zebrafish, chicken embryos, mouse stem cells, and human disease demonstrates the preservation of an ancient intron-mediated gene regulation system in eukaryotes. In these animal models, the intron-derived miRNA produces an RNAi-like gene silencing effect. We herein provide the first

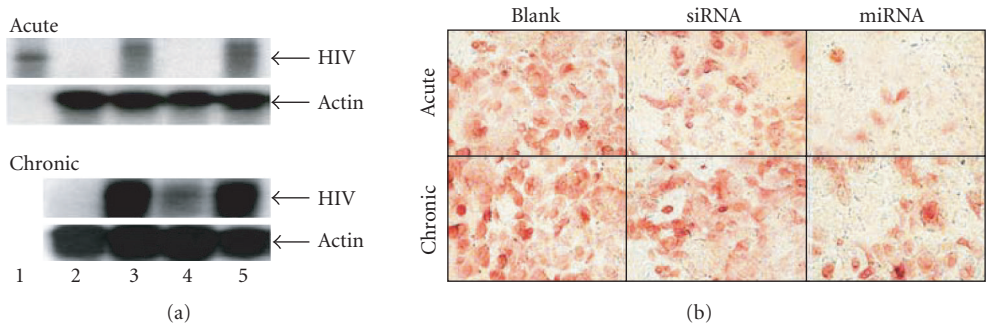

FIGURE 5: Silencing of HIV-1 genome replication using anti-*gag/pro/pol* miRNA transfection into $CD4^+$ T lymphocytes isolated from the acute and chronic phases of AIDS infections. (a) Northern blot analysis showed about 98% and 70% decreases of HIV genome in the acute and chronic infections after miRNA treatments (lane 4), respectively. No effect was detected in the T cells transfected with miRNA* targeting the same *gag/pro/pol* region of the viral genome (lane 5). The size of pure HIV-1 provirus was about 9,700 nucleotide bases (lane 1). RNA extracts from normal noninfected $CD4^+$ T lymphocytes were used as a negative control (lane 2), whereas those from HIV-infected T cells were used as a positive control (lane 3). (b) Immunostaining for HIV p24 marker confirmed the results in (a). Since the ex vivo HIV-silenced T lymphocytes were resistant to any further infection by the same strains of HIV, they may be transfused back to the donor patient for eliminating HIV-infected cells.

evidence for the biogenesis and function of intronic miRNA in vivo. Given that evolution has given rise to more complexity and more variety of introns in higher animal and plant species for the task of coordinating their vast gene expression libraries and interactions, dysregulation of these miRNAs due to intronic expansion or deletion will likely cause genetic diseases, such as myotonic dystrophy and fragile X mental retardation. Thus, gene expression produces not only gene transcripts for its own protein synthesis but also intronic miRNA, capable of interfering with the expression of other genes. Thus, the expression of a gene results in gain-of-function of the gene and also loss-of-function of other genes, with complementarity to the mature intronic miRNA. An array of genes can swiftly and accurately coordinate their expression patterns through the mediation of their intronic miRNAs, bypassing the time-consuming translation process in quickly changing environments. Conceivably, intron-mediated gene regulation may be as important as the mechanisms by which transcription factors regulate gene expression. It is likely that intronic miRNA is able to trigger cell transitions quickly in response to external stimuli without such tedious protein synthesis. Undesired gene products are reduced by both transcriptional inhibition and/or translational suppression via miRNA regulation. This could enable a rapid switch to a new gene expression pattern without the need to produce various transcription factors. This regulatory property of miRNAs may have modulated ancient gene even before the emergence of proteins in the post-RNA world. Considering the variety of microRNAs and the complexity of genomic introns, a thorough investigation of miRNA variants in the human genome will markedly improve the understanding of genetic diseases and also the design of miRNA-based drugs. Learning how to exploit such a novel gene regulation system for future therapeutic applications will be a great challenge.

ACKNOWLEDGMENT

This study was supported by NIH/NCI Grant CA-85722.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 95270, Pages 1–10
DOI 10.1155/JBB/2006/95270

Review Article

Computational Prediction of MicroRNAs Encoded in Viral and Other Genomes

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Received 1 February 2006; Revised 5 April 2006; Accepted 2 May 2006

We present an overview of selected computational methods for microRNA prediction. It is especially aimed at viral miRNA detection. As the number of microRNAs increases and the range of genomes encoding miRNAs expands, it seems that these small regulators have a more important role than has been previously thought. Most microRNAs have been detected by cloning and Northern blotting, but experimental methods are biased towards abundant microRNAs as well as being time-consuming. Computational detection methods must therefore be refined to serve as a faster, better, and more affordable method for microRNA detection. We also present data from a small study investigating the problems of computational miRNA prediction. Our findings suggest that the prediction of microRNA precursor candidates is fairly easy, while excluding false positives as well as exact prediction of the mature microRNA is hard. Finally, we discuss possible improvements to computational microRNA detection.

INTRODUCTION

Since 2000 the interest in microRNAs (miRNAs) and their role as gene expression regulators has grown immensely. Lee et al were the first to identify such a small regulator: the lin-4 RNA in *Caenorhabditis elegans* [1]. It has been shown that the 21 nt lin-4 RNA represses mRNA and controls part of the *C elegans* larval development [1, 2]. The next small regulatory RNA to be discovered was the let-7, which controls a later stage in the development of *C elegans* [3, 4]. The lin-4 and let-7, previously known as small temporal RNAs (stRNAs), are today recognized as the first of a large class of small regulatory noncoding RNA molecules now called microRNAs [5]. This class of molecules is not limited to development but regulates a wide range of biological processes [6]. The microRNAs have been reported to be encoded within noncoding regions of genomes [5, 7, 8], and within protein coding genes [9] as well as noncoding genes [10].

Primary precursor miRNAs (pri-miRNAs) are long transcripts that contain one or more miRNA precursors (pre-miRNAs) [11]. Subsequently the pri-miRNA is cut by the Drosha enzyme into one or more $\sim$ 70 nt long pre-miRNA stem-loop (hairpin) structure(s) while still in the nucleus [12]. The pre-miRNAs are transported by exportin-5 to the cytoplasm [13–15], where they are cut by the RNase III Dicer enzyme into active $\sim$ 22 nt long miRNAs [16–18] (Figure 1). Usually only one side of the stem encodes a mature miRNA [5, 19], however the process of selecting the side and region of the pre-miRNA that becomes a mature miRNA is still not fully understood. The mature miRNAs are then incorporated as subunits of the micro-ribonucleoproteins (miRNPs) [20]. The miRNP is able to repress the transcription of target mRNAs by binding to or cleaving the mRNA. Thus the miRNA is capable of posttranscriptional regulation [1–4, 21–23]. Such a posttranscriptional silencing complex is often called an miRNA-initiated (or associated) RISC complex (RNA-induced silencing complex), and is very similar to the small interfering RNA-initiated RISC complexes [21, 24]. Detailed descriptions of the stepwise maturation of microRNAs are presented by Chen and Meister [25] and by Bartel [26].

Different miRNAs have been detected in a variety of organisms; including 114 *C elegans* miRNAs, 326 human miRNAs, and a total of 35 virus-encoded miRNAs (miRBase release 7.1, October 2005) [27, 28]. It is estimated that as much as 30% of human genes are regulated by miRNAs [29, 30].

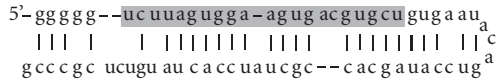

Figure 1: Sequence and structure of a pre-miRNA molecule encoding a miRNA detected by Pfeffer et al [33] from the Epstein-Barr virus. The mature 21 nt EBV mir-BART1 miRNA sequence is shown on a grey background.

COMPUTATIONAL DETECTION OF miRNAs IN SELECTED ORGANISMS

Until 2003 miRNAs were identified almost exclusively by experimental molecular biology [31] because there were few computational miRNA prediction tools available (except for homology searches).

According to Lai et al [32], three observations suggest that computational miRNA prediction approaches will be feasible. "First, miRNAs are generally derived from precursor transcripts of 70–100 nucleotides with extended stem-loop structure. Second, miRNAs are usually highly conserved between the genomes of related species. Third, miRNAs display a characteristic pattern of evolutionary divergence."

Already in 2001 Lee and Ambros used both bioinformatics and cDNA cloning to identify potential *C elegans* miRNAs [7]. They searched the *C elegans* genome for sequences conserved in *C briggsae* that also had characteristic pre-miRNA features and a secondary structure similar to lin-4 and let-7, as computed by the mfold program [37]. They reported 15 novel miRNAs, of which two were the results of the computational screening, while the rest were derived from the cDNA cloning. Table 1 contains an overview of computational miRNA prediction studies.

Another computational tool for miRNAs identification is MiRscan, described by Lim et al in 2003 [31]. MiRscan was designed to identify miRNA genes conserved between genomes, and was initially applied to *C elegans* and *C briggsae*. MiRscan was utilized together with extensive sequencing of clones, resulting in the detection of 30 additional miRNAs.

MiRscan starts out with two closely related genomes A and B. It scans genome A for sequences that could form hairpin structures and then checks if the sequences are conserved in genome B. This initial search aims at capturing most of the homologous pre-miRNAs in the two genomes. The program uses the captured miRNAs that are already experimentally verified as a training set, and then computes a score for all the initially recognized sequences.

Lim et al found 35 novel miRNA candidates in *C elegans* using MiRscan, of which 16 were experimentally validated. In addition, the program used a detection threshold that would have identified half (29) of the known (58) miRNAs. This implies that in the worst case, the MirScan program would have a sensitivity of 0.70 for miRNAs detection in this study.

Lim et al also showed that the accuracy of MirScan is lower than for programs designed to detect one special type of RNA, such as tRNAs [38], but on the other hand it is at least as good as general computer algorithms for detection of bacterial ncRNAs [39–41]. Due to the homology criterion of MiRscan, it may be questionable whether this program is suitable for the detection of viral miRNAs as there are reports on viral miRNAs not being conserved across species [33], as well as reports on the opposite [36]. MiRscan has proved itself able to detect a large number of miRNAs in vertebrate genomes with a detection sensitivity of 0.74 [42].

In May 2003, Ambros et al reported on the testing of different methods for the detection of miRNAs in *C elegans* [34]. This study was a follow up to their 2001 study, when only 10% of the *C briggsae* genome was available [7]. Two computational approaches were based on sequence similarities and stem-loop structure features, but used slightly different algorithms. The algorithms were complementary in the way that the methods uniquely identified miRNAs and in total these two approaches identified 9 new miRNAs. Combined with a third approach, cDNA cloning followed by Northern blots, they discovered a total of 21 novel miRNAs.

Others have also screened the *C elegans* genome for miRNAs using computational approaches based on hairpin structure searches, secondary structure predictions, and interspecies sequence conservation. Grad et al suggested 214 miRNA candidates of which 14 were confirmed by expression analysis [43].

In 2003 Lai and colleagues described a computational method for miRNA identification in *Drosophilia* [32]. The approach was named miRseeker, and the initial step was to search the euchromatic DNA sequences of *D melanogaster* and *D pseudoobscura* for transcripts potentially forming stem-loop structures and having a "pattern of nucleotide divergence characteristic of known miRNAs." Subsequently they considered the conservation of this sequence in more distantly related insects. Lai et al started by aligning 24 pre-miRNA sequences from the two *Drosophilia* species and found the degree of conservation to be higher than in protein coding regions. The candidates were then subjected to a stricter selection procedure due to the many conserved possible pre-miRNA stem-loops found. Further analysis proved that most divergence in the orthologous *Drosophila* miRNAs originated in loop-mutations. In more diverged species only the 21–24 nt mature miRNAs were found to be preserved. The algorithm consists of three steps. Initially it aligns all *D melanogaster* and *D pseudoobscura* intronic and intergenic regions. It then slides a window along the conserved regions and uses mfold [37] to estimate the free energy of potential secondary structure formed by the sequence in the window. A minimum arm length of 23 nt was required as well as a free energy of at most −23.0 kcal/mol for one isolated miRNA precursor arm. Both strands of the DNA sequence in the sliding window were mfolded. Additional scoring of the stem-loops was also applied. Finally, miRseeker attempts to fit all the remaining miRNA-precursor candidates into one of six stem-loop pattern classes defined by the initial 24 pre-miRNA training set. This procedure left 208 miRNA candidates, including 18 (75%) from the training set among the 124 highest scoring candidates. Out of the 208 candidates 42 were also found to be conserved (by sequence and structure) in a third species. In a selection of 38 candidates, 24 were confirmed as novel miRNA genes (20/27 of those conserved in a

Table 1: Overview, in chronological order, of approaches and results of selected miRNA detection studies. Computational and experimental approaches used as well as the total number of predicted candidates and verified miRNAs are indicated for each study.

Reference	Genome(s)	Stem-loop	Homology	Folding (free energy)	Experimental	Novel candidates (comp/exp)	Novel verified (comp/tot)
Lee and Ambros [7]	*C elegans*	X	X	X	X	40/38 (only 53 tested)	2/13
Lim et al [31]	*C elegans*	X	X	—	X	35/NA	16/30
Ambros et al [34]	*C elegans*	X	X	X	X	407/NA	9/21
Lai et al [32]	*D melanogaster*	X	X	X	—	166/0 (only 38 tested)	24/24
Pfeffer et al [35]	Epstein-barr virus (EBV)	—	—	—	X	0/NA	5/5
Pfeffer et al [33]	Human cytomegalovirus (HCMV)*	X	—	X	X	11/NA	5/9
Grey et al [36]	Human cytomegalovirus (HCMV)	X	X	—	—	10/0	2/2

*One selected genome of a range of herpesviruses studied.

third species and 4/11 of the *Drosophila* specific candidates). Lai and colleagues also estimated miRNAs to make up about 1% of the total amount of genes in the *Drosophila* genomes (94–124 miRNA genes), while Grad et al estimated *C elegans* to code for 140–300 miRNA genes [43]. As a concluding remark, Lai et al state that their algorithm excludes at least one known miRNA (miR-100).

Another study exploiting both characteristic miRNA features and sequence conservation was developed by Wang et al [44]. This approach was used in their search for *Arabidopsis thaliana* miRNAs. Their prediction identified 63% of known *Arabidopsis* miRNAs, and they claim identification of 83 novel miRNAs, of which 25 were verified. The computer algorithm evaluated possible miRNA precursors based on their stem-loop structure, the GC content of the mature miRNA, the loop length, mismatches in the stem containing the mature miRNA and the conservation of mature miRNA sequence in the *Orysa sativa* genome. Interestingly, 15 of the 19 already known unique *Arabidopsis* miRNAs have a loop ranging from 20–75 nt, which is much longer than in the known viral miRNAs [19, 33, 35, 36].

In plants, the alignment of the miRNA and its target mRNA contains few mismatches. This fact has been successfully exploited in combination with typical miRNA feature and conservation searches, as described above, in a search for *Arabidopsis thaliana* miRNA [45].

Yet another project combining bioinformatics and experimental biology in the quest for *A thaliana* and *Nicotiana tabacum* miRNA chose a "reverse" approach [46]. Billoud first created a cDNA library of all short *N tabacum* RNAs, then computational methods were used to identify potential miRNAs. Their pattern matching program, Patbank, was used for finding homologues and their MIRFOLD program was used to check for possible miRNA secondary structures.

In this context, the microHarvester should be mentioned as it is a useful web service designed to detect miRNA homologues in any set of sequences, given an miRNA precursor [47]. The microHarvester is filter based and uses the conservation patterns of the microRNAs combined with BLAST [48], Smith-Waterman [49], and RNAfold [50].

Wang et al presented a new computational tool in 2005 designed to search for homologues and paralogues of known miRNAs; miRAlign [51]. It is claimed that miRAlign outperforms all earlier programs of this kind, due to a less strict conservation search, the ability to take more structural properties into account, as well as its capability to create structural alignments based on a single miRNA. It should be noted that miRAlign is tested primarily on animal data. It was able to detect 59 miRNA candidates in *Anopheles gambiae* of which 37 has later been reported in the MicroRNA registry [27, 28].

COMPUTATIONAL DETECTION OF miRNAs IN VIRAL GENOMES

The first miRNAs detected in a viral genome were reported in Science 2004 [35]. Pfeffer and colleagues recorded the small RNA profile of Epstein-Barr virus (EBV) positive cells. They detected several expressed miRNA genes in EBV, and given the function of miRNAs they concluded that they had identified regulators of host and/or viral gene expression. The detection of these 5 novel miRNAs was made by cloning of small RNAs from EBV-infected cells. 4% of the small RNAs originated from EBV. The 5 novel EBV miRNAs were detected by Northern blotting. One miRNA was found in the 5′ UTR, one in the coding sequence, and one in the 3′ UTR of the same gene, *BHRF-1*. The last two miRNAs are from a cluster in the intronic regions of the *BART* gene. The miRANDA algorithm was used in their prediction of mRNA targets, a method developed for detecting miRNA targets in *Drosophila* [52]. Several host and/or EBV mRNA targets were found for every miRNA. The majority of the target mRNAs have more then one miRNA binding site.

In 2005 Pfeffer et al reported on the identification of several miRNAs in the herpesvirus family [33]. Their study combined a new computational method for miRNA prediction with a cloning approach similar to the one used in their initial discovery of viral miRNAs [35]. They were able to predict miRNAs in many large DNA viruses, but they were unable to predict or experimentally identify miRNAs in small RNA viruses or retroviruses. Another important finding in this study was that the EBV miRNAs neither have any significant sequence similarity with host miRNAs, nor do they seem to be conserved in the herpesvirus family. This observation indicated that methods depending on cross-species sequence conservation such as MiRscan and miRseeker,

described above, are not well suited for prediction of viral miRNAs. The computational approach developed by Pfeffer and colleagues was based on defining a set of properties of known miRNA precursor stems and subsequently training a support vector machine (SVM) to separate known pre-miRNAs from stem-loops unlikely to code for miRNAs. The SVM was then applied on the set of all genomic regions potentially forming a stem-loop secondary structure. The SVM reported predictions based on a chosen threshold that resulted in the detection of 71% of the true pre-miRNAs from the training set with only 3% false positives. Their program also had a method for ranking the candidates with a score above the threshold; this method is independent of the SVM threshold score. Disregarding the direction of transcription, Pfeffer et al made 23 unique predictions of which 14 (61%) were experimentally verified. One should keep in mind that some of the predicted miRNAs can be very hard to detect as they may be expressed only under rare conditions.

Further studying the expression of the EBV *BHRF-1* gene and its miRNAs, Pfeffer and colleagues suggest that viruses are able to simultaneously transcribe both miRNAs and mRNA from the same region. Pfeffer et al also suggest that their conclusions support the view of independent miRNA evolution in viruses, as viral miRNAs seem to lack sequence conservation. In addition, most miRNAs are transcribed by pol II [53], while viral miRNAs may also be transcribed by pol III [25, 33].

Almost at the same time as Pfeffer et al published their results [33], Cai et al published a paper on the detection of miRNAs in the human pathogenic Kaposi's sarcoma-associated herpesvirus (KSHV) [54]. They reported the detection of 11 distinct miRNAs, of which all were expressed in latent KSHV infected cells. These 11 miRNAs were detected by cloning small RNAs followed by RT-PCR and Northern blot analyses. MirBase (release 7.1, October 2005) [27, 28] lists 12 KSHV miRNAs, of which 10 were identified in both studies, while both Pfeffer et al and Cai et al report one additional unique miRNA.

Grey et al developed a computational method based on pre-miRNA stem-loop properties and combined it with stem-loop conservation [36], despite the findings by Pfeffer et al about lack of sequence conservation for viral miRNAs, but in line with the findings in primates [55]. Grey and colleagues studied the closely related human and chimpanzee cytomegaloviruses (HCMV and CCMV). First, all conserved stem-loop structures scoring better than a 60% similarity threshold were detected. The resulting 110 highly conserved stem-loop sequences were then run through the MiRscan program [31]. MiRscan then suggested 13 high-scoring candidates. Northern blot analysis was used on total RNA harvested at different time points for transcription verification. Five of the 13 candidates were expressed during infection, and three of these were among the ones detected by Pfeffer et al. All but one of the miRNAs found in the study by Pfeffer et al but not identified in the study by Grey et al were conserved in CCMV and had a MiRscan score above the threshold. The reason they were not detected was the initial stem-loop finder algorithm.

The miRNAs of the simian virus 40 (SV40) has also been studied [19]. Sullivan et al created a computer program called VirMir that identifies miRNA precursor candidates in small genomes (max 300 kbp). The VirMir program utilizes the RNAfold package [50]. Sullivan and colleagues ended up with two candidates out of which one region produced a suitably sized pre-miRNA that was detected by a Northern blot. The detected miRNA precursor was found to be a member of a seemingly small fraction of the miRNA precursor family, namely, those producing one mature miRNA from each stem of the precursor hairpin. Interestingly, they also discovered that both of these miRNAs are acting on the same target mRNA.

Bennasser et al argue that there are 5 likely miRNA candidates in the human immunodeficiency virus (HIV-1) [56]. Attempts to validate the candidates were in progress, but all of the miRNA candidates were found to have several cellular mRNA targets by a rule based target finder algorithm. As small-interfering RNAs (siRNAs) are somewhat related to miRNAs due to the fact that their pathways partially overlap and both become part of a RISC complex [21, 24], it is worth mentioning that the HIV-1 genome encodes an siRNA [57]. So there is evidence that viruses can encode both miRNAs and siRNA. The existence of both viral miRNAs and siRNAs was also suggested by Lu and Cullan in their paper on the adenovirus VA1 [58].

A COMPUTATIONAL SEARCH FOR EBV miRNA PRECURSORS

In 2004 we investigated the challenges in computational detection of miRNAs encoded in the EBV genome. The EBV genome sequence (NC_001345) was retrieved from NCBI, and then the sRNAloop program [43] (parameters: hairpin structure no more than 75 nt, loop longer than 3 nt, score threshold 22) was used to scan the entire genome for potential miRNA precursors. A total of 148 candidates were found, including all the five known EBV miRNAs. We kept only one copy of the candidates appearing more than once in the genome, narrowing down the number of candidates to 70. Potential miRNA precursors inside coding regions were not excluded. We then used mfold [37] to estimate the free energy of the entire precursors, using the web service (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). The free energy estimates for the five known EBV miRNAs ranged from -25 kcal/mol to -33.8 kcal/mol. We kept approximately 40 candidates having a free energy less than -24.5 kcal/mol, which is about the same threshold as used in the study by Lai et al [32].

We then ranked the candidates as follows: the candidates from nonrepeat noncoding regions or hypothetical protein coding regions were ranked first, followed by candidates in known protein coding regions, and finally the remaining candidates. All of the five known pre-miRNAs were among the top ten candidates. Based on these criteria we selected the top 14 candidates for further studies, including the 5 known miRNAs. This leaves 9 novel predictions, as shown in Table 2, the according secondary structure predictions can be found in Figure 2. Attempts to experimentally verify either

Table 2: Computationally predicted miRNA precursor candidates from the Epstein-Barr virus (this study), ranked according to our criteria. The free-energy estimates were computed by the online mfold [37] version of December 2004. All predicted secondary structures can be found, according to the given letter, in Figure 2.

Name,[1] structure	Position	Direction	Length	Free energy[2]	Sequence	Notes
PMRP 1, a	53263–53332	+	69	−25.4 kcal/mol	AUAACCUAUAGGUU-AUUAACCUAGUGGU-GGAAUAGGGUAUUG-CAGCUGGGUAUAUA-CCUAUAGGUAUAU	Intergenic region, poly A signals upstream
PMRP 2, b	6838–6912	−	74	−32.7 kcal/mol	UACGUCACGGUUGUA-GGCGGGGUUAAGCGU-GCAUCUUCUGGGAUG-CAACGUUAAGCCCCG-UUUAGGUGGAACUG	Intergenic region
PMRP 3, c	9041–9116	+	74	−29.8 kcal/mol	AUGCUUCCCGUUGG-GUAACAUAUGCUAUU-GAAUUAGGGUUAGUC-UGGAUAGUAUAUACU-ACUACCCGGGAAGCAU	Intergenic region, poly A signals upstream, promoter at 8573
PMRP 4, d	61262–61333	+	71	−43.8 kcal/mol	UGCCAUCAUCCCCUG-CUUGGGACCCGACCG-CACUUGCAUGCGGCC-GGUGGUCCUGCGGGG-GGUGACGGUCA	Inside a hypothetical protein coding region
PMRP 5, e	1898–1973	−	75	−32.7 kcal/mol	CUCCUGACGCUGAGG-CCUGGGAUCGUUGUU-GGUGCCACGCAGCGC-CACUAGCAGCAGGUU-CUCAGCAAUCAGGGG	Inside a coding region
PMRP 6, f	7408–7483	+	75	−24.6 kcal/mol	CCACUCUACUACUGG-GUAUCAUAUGCUGAC-UGUAUAUGCAUGAGG-AUAGCAUAUGCUACC-CGGAUACAGAUUAGG	Intergenic repeat region[3]
PMRP 7, g	7454–7526	+	72	−25.4 kcal/mol	UAGCAUAUGCUACCC-GGAUACAGAUUAGGA-UAGCAUAUACUACCC-AGAUAUAGAUUAGGA-UAGCAUAUGCUA	Intergenic repeat region
PMRP 8, h	7929–8003	+	74	−29.4 kcal/mol	AUAGCAUAUGCUACC-CAGAUAUAGAUUAGG-AUAGCCUAUGCUACC-CAGAUAUAGAUUAGG-AUAGCAUAUGCUAU	Intergenic repeat region, promoter at 7888
PMRP 9, i	151510–151584	+	74	−34.0 kcal/mol	UUGGUGGGACCUGAU-GCUGCUGGUGUGCU-GUAAAUAAGUGCCUA-GCACAUCACGUAGGC-ACCAGGUGUCACCAG	Intergenic repeat region
BHRF 1-1, j	53754–53829	+	75	−27.9 kcal/mol	CUCCUUAUUAACCUG-AUCAGCCCCGGAGUU-GCCUGUUUCAUCACU-AACCCCGGGCCUGAA-GAGGUUGACAAGAAG	Holds known miRNA; BHRF 1-1

Table 2: continued.

Name,[1] structure	Position	Direction	Length	Free energy[2]	Sequence	Notes
BHRF 1–2, k	55131–55206	+	75	−32.1 kcal/mol	CCCCACUUUUAAAUU-CUGUUGCAGCAGAUA-GCUGAUACCCAAUGU-UAUCUUUUGCGGCAG-AAAUUGAAAGUGCUG	Holds known miRNA; BHRF 1–2[4]
BHRF 1–3, l	55248–55323	+	75	−25.0 kcal/mol	UGGUGUUCUAACGGG-AAGUGUGUAAGCACA-CACGUAAUUUGCAAG-CGGUGCUUCACGCUC-UUCGUUAAAAUAACA	Holds known miRNA; BHRF 1–3
BART 1, m	151631–151706	+	75	−33.8 kcal/mol	CGUGGGGGGUCUUAG-UGGAAGUGACGUGCU-GUGAAUACAGGUCCA-UAGCACCGCUAUCCA-CUAUGUCUCGCCCGG	Holds known miRNA; BART 1
BART 2, n	153197–153272	+	75	−30.8 kcal/mol	UUCCAGACUAUUUUC-UGCAUUCGCCCUUGC-GUGUCCAUUGUUGCA-AGGAGCGAUUUGGAG-AAAAUAAACUGUGAG	Holds known miRNA; BART 2

[1]The novel candidates are named PMRP (possible micro RNA precursor) 1 through 9.
[2]Energy calculations made using mfold [37].
[3]Mfold suggests two possible secondary structures for this sequence, only one structure is shown.
[4]Pfeffer et al [35] state that this hairpin structure gives two mature miRNAs, one from each stem-arm, the other is named BHRF 1–2*.

the 5 known miRNAs or the 9 new candidates were unsuccessful. Several possible human and EBV target mRNAs were predicted for the 9 novel pre-miRNA candidates (data not shown) using a ParAlign [59] sequence similarity search with the predicted stem sequences and a set of rules similar to the ones used by the miRANDA algorithm [52]. A schematic view of our approach can be found in Figure 3.

DISCUSSION

It is important to assess the significance of viral miRNA-induced posttranscriptional gene regulation in an infected cell. In *C elegans*, miRNAs play vital roles during development [3, 4], while such a critical role for miRNAs has not yet been discovered in viruses. Sullivan et al argue that the importance of the EBV miRNAs in viral mRNA regulation is uncertain, while claiming a more important role of the SV40 miRNA, which they have proven to reduce the cytotoxic T-lymphocyte susceptibility and also reduce local cytokine release [19]. The homology findings of Grey et al indicate that the viral miRNAs have not evolved independently [36], suggesting a more significant role than implied by theories of independent evolution.

The importance of further miRNA knowledge is illustrated by the successful use of miRNA expression profiles to classify human cancers [60], as well as data indicating that many human miRNAs are located in regions frequently associated with cancer [61].

Our study clearly indicates that predicting pre-miRNA structures seems reasonably easy apart from deciding on a score threshold for candidates. The most challenging task is to predict the accurate position of the mature miRNA within the precursor. The most promising strategy for predicting novel miRNAs in viruses appears to be a combination of the conserved stem-loop search by Grey et al and the precursor miRNA feature searches used in the Grey and Pfeffer studies. Grey et al suggest a refinement of the stem-loop finder to improve the search results as it excluded true positives that would have been accepted by the later stages of the algorithm. A broader search for stem-loop structures is also anticipated by the reports by Wang et al [44] of much longer loops (20–75 nt) in *A thaliana* than in the loops in the known HMCV miRNAs (4–12 nt) [33, 36].

Algorithms might also be improved by exploiting the findings of Berezikov et al [55]; while miRNAs stems show strong conservation and the loops vary in their degree of conservation, the miRNA precursors' flanking regions show a striking drop in conservation. This conservation profile can be used for phylogenetic shadowing [62], a technique for sequence comparison between closely related species. This approach was used to predict and identify several primate miRNAs [55].

Introducing a search for miRNA targets [29, 52, 63–67] at an earlier stage of the algorithm could also improve the results. In most miRNA detection approaches this is often a final separate part [44, 45]. We suggest that including an

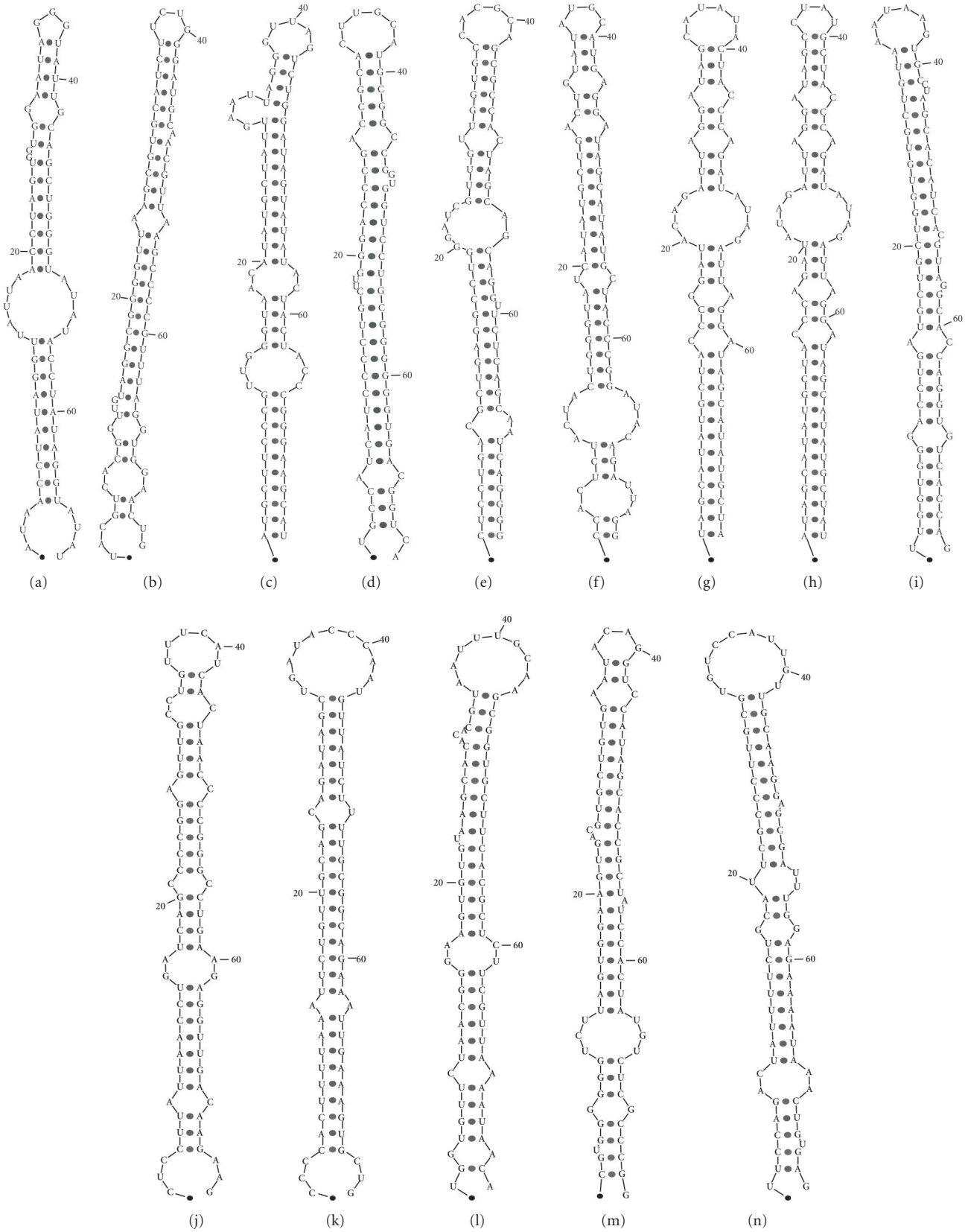

Figure 2: (a)–(i) The predicted structure of the nine top scoring novel miRNA precursor candidates. (j)–(n) The predicted structure of the five known EBV miRNA precursors.

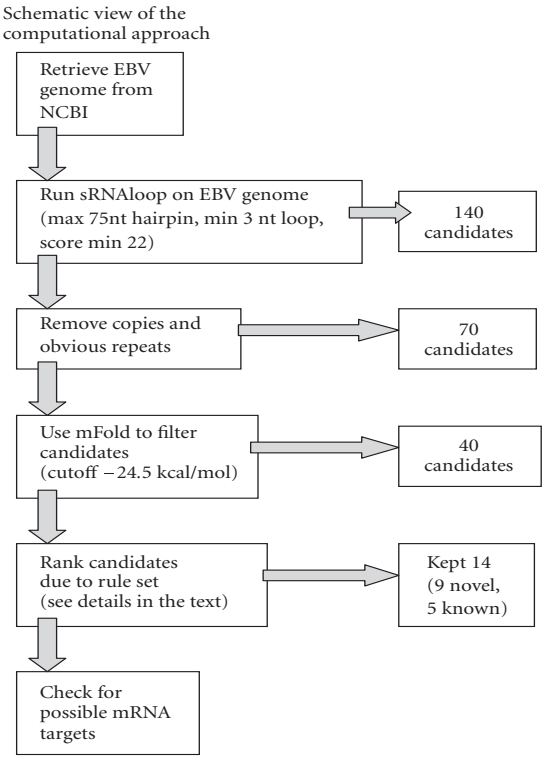

Figure 3: Schematic view of the computational approach.

miRNA regulatory module (MRM) [68] search at an early stage could be a valuable improvement.

Concerning experimental approaches and verification it should be noted that miRNA candidates found to originate from within exons are often regarded as cloning artefacts and therefore discarded. However, as stated by Berezikov et al, there is no experimental evidence excluding miRNAs candidates in these regions [55]. Furthermore, there is evidence indicating that a region coding for both an miRNA and a protein can be used almost simultaneously for miRNA and protein production [54]. A large portion of the currently known miRNAs have emerged as a result of cloning, but cloning approaches are likely to be biased towards abundant miRNAs [43].

Current computational methods are useful tools for identifying miRNA candidates, however before better methods have been developed, we still need to verify candidates using Northern blots.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 64347, Pages 1–12
DOI 10.1155/JBB/2006/64347

Research Article

Dicer-Derived MicroRNAs Are Utilized by the Fragile X Mental Retardation Protein for Assembly on Target RNAs

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Received 27 April 2006; Accepted 30 May 2006

In mammalian cells, fragile X mental retardation protein (FMRP) has been reported to be part of a microRNA (miRNA)-containing effector ribonucleoprotien (RNP) complex believed to mediate translational control of specific mRNAs. Here, using recombinant proteins, we demonstrate that human FMRP can act as a miRNA acceptor protein for the ribonuclease Dicer and facilitate the assembly of miRNAs on specific target RNA sequences. The miRNA assembler property of FMRP was abrogated upon deletion of its single-stranded (ss) RNA binding K-homology domains. The requirement of FMRP for efficient RNA interference (RNAi) in vivo was unveiled by reporter gene silencing assays using various small RNA inducers, which also supports its involvement in an ss small interfering RNA (siRNA)-containing RNP (siRNP) effector complex in mammalian cells. Our results define a possible role for FMRP in RNA silencing and may provide further insight into the molecular defects in patients with the fragile X syndrome.

INTRODUCTION

MiRNAs form a class of small regulatory RNAs (∼ 21–23 nt) involved in guiding translational repression or cleavage of mRNAs [1]. Biosynthesis of miRNAs is initiated upon transcription of miRNA-encoding genes into primary miRNAs (pri-miRNAs) by RNA polymerase II [2]. Pri-miRNAs are then processed by the nuclear ribonclease (RNase) III Drosha into miRNA precursors (pre-miRNAs) [3]. Following export to the cytoplasm via Exportin 5 [4], the imperfectly paired stem-loop pre-miRNAs are cleaved into miRNA:miRNA* duplexes by the RNase III Dicer [5–8]. Based upon the stability of the base pairs at the 5′ ends of the two strands [9], the mature miRNA will be incorporated into the RNA-induced silencing complex (RISC) [10], whereas the opposite miRNA* strand is encountered much less frequently and is presumably degraded [1]. The mRNA specifically recognized by the RISC will either be cleaved or translationally repressed, depending on whether the miRNA:mRNA pairing is perfect or not [1].

Hundreds of miRNAs have been identified in *Drosophila*, *C elegans* [11], *A thaliana* [12], zebrafish, mice, human cells [13–15], and viruses [16]. The ability of some of these miRNAs to regulate translation of specific mRNAs has been demonstrated experimentally. For example, cel-let-7 silences *lin-41* mRNA through binding to its 3′ untranslated region (UTR) in *C elegans* [17], whereas miR-196 mediates cleavage of the *HOXB8* mRNA in mouse embryos [18]. A mechanistic insight into miRNA-mediated translational repression was recently provided, as endogenous let-7 miRNPs were shown to inhibit translation initiation [19]. mRNA functional regulation by miRNAs has been involved in various cellular processes, such as leaf morphogenesis in plants, developmental timing and left/right asymmetry in nematodes, cell proliferation and apoptosis in flies, and hematopoietic cell differentiation in mice [1].

In humans, loss of expression of the *FMR1* (fragile mental retardation 1) gene product, the fragile X mental retardation protein (FMRP), is the etiologic factor of the fragile X syndrome, the most frequent cause of inherited mental retardation [20, 21]. FMRP is an RNA-binding protein that contains two K-homology (KH) domains and an RGG box and is involved in RNA regulation of translation, RNA transfer, and local modulation of synaptic mRNA translation.

However, its exact roles are still unclear and the mechanisms by which it controls translation are poorly understood. FMRP has been reported to behave as a negative regulator of translation both *in vitro* and in vivo [22–26], and it is deduced that the miRNA-guided RNA silencing pathway could be a cellular process through which FMRP could regulate translation of target mRNAs.

Indeed, a relation between FMRP and components of the RNAi machinery was uncovered. The ortholog of FMRP (dFMR1) was found to be associated with the effector RISC as well as miRNAs in *Drosophila* S2 cells [27, 28]. In mammalian cells, FMRP has been reported to be part of a ribonucleoprotein (RNP) complex with miRNAs and Argonaute 2 (Ago2) [29]. However, how FMRP functions in miRNA-mediated translational control remains unknown.

In this study, we have used recombinant proteins to show that human FMRP can accept miRNAs derived from Dicer cleavage and facilitate the formation of specific miRNA: target transition complexes in vitro. Reporter gene silencing assays, using various small regulatory RNAs, revealed the requirement of FMRP for efficient RNAi in vivo. The results obtained with single-stranded (ss) antisense siRNA also support its involvement in an ss siRNP effector complex in mammalian cells.

MATERIALS AND METHODS

Protein expression, purification, and analysis

Recombinant Dicer [6], FMRP deleted variant ΔKHT, FMRP mutant I304N, and FXR1P [30] proteins were expressed and purified as previously reported. Immunoblot analysis was performed with previously described antibodies recognizing FMRP [30] and FXR1P [31] proteins, and the immunoreactive proteins visualized with peroxydase-labeled affinity-purified goat anti-rabbit or mouse IgG secondary antibody using Western Lightning Chemiluminescent Reagent (PerkinElmer).

Preparation of RNAs

For in vitro experiments, all RNA substrates contained 5′-p and 3′-OH ends (for complete sequence and structure information, see Supplementary text available online at DOI 10.1155/JBB/2006/64347). Synthetic RNAs (Dharmacon) devoid of 5′-p were labeled at their 5′ end using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32} P] ATP (PerkinElmer), and purified by denaturing PAGE. The following siRNA duplexes were used: CLP siRNA (5′-pAGGAGGUCGUACAGAAUUUdtdt) and Dicer siRNA (5′-pUGCUUGAAGCAGCUCUGGAdtdt).

Target RNAs ($\sim$ 60 or $\sim$ 100 nt), encompassing the miRNA/siRNA binding sites and flanking regions, were synthesized from DNA templates by in vitro transcription using the T7 RNA polymerase (Ambion) and purified by denaturing PAGE. Templates were obtained either by annealing DNA oligonucleotides or by introducing a T7 RNA polymerase promoter by PCR followed by gel purification. The following target RNAs were used: CLP target RNA (nt 414–514, Acc no L54057), Dicer target RNA (nt 353–453, Acc no AJ132261), *lin-41* target RNA (nt 4100–4162, Acc no AF195610), and *HOXB8* target RNA (nt 2237–2297, Acc no X13721).

Prior to use, miRNAs and target RNAs were denatured at 90°C for 2 min and chilled on ice. Pre-miRNAs and miRNA:miRNA* were renaturated, and complementary strands of siRNA duplexes annealed, by incubation at 95°C for 5 min, and then slowly cooled down to room temperature.

For cell transfection, the siRNA duplexes siRluc (5′-GGCCUUUCACUACUCCUACdtdt) and siCtl (5′-GUAUAAUACACCGCGCUACdtdt) were used, whereas the ss antisense siRNAs asRluc (5′-GUAGGAGUAGUGAAAGGCCdtdt) and asCtl (5′-GUAGCGCGGUGUAUUAUACdtdt) were phosphorylated in vitro using T4 polynucleotide kinase, followed by ethanol precipitation, prior to use.

Dicer RNase assays

^{32}P-labeled pre-miR-31 RNA (40000 cpm) was incubated in the absence or presence of recombinant Dicer (50–400 ng) and/or FMRP (0.18–2.8 μg) with $MgCl_2$ (5 mM) at 37°C for 1 h, as described [6]. The samples were analyzed by denaturing PAGE and autoradiography. For the experiments with FMRP, the samples were treated with 40 μg proteinase K (Ambion) at 55°C for 45 min prior to analysis. RNA derived from Dicer cleavage was gel-purified and incubated with increasing amounts of recombinant FMRP (0.35–1.4 μg), without or with BSA (20 μg), for 30 min on ice prior to EMSA analysis.

EMSA

^{32}P-labeled human GFP siRNA (0.25 pmol), hsa-miR-196a-1 miRNA:miRNA* duplex (0.50 pmol), or miRNA (0.50 pmol) (40000 cpm) was incubated in the absence or presence of recombinant FMRP (0.35–1.4 μg) for 30 min on ice prior to electrophoretic mobility shift assay (EMSA) analysis, as described [18]. FMRP-RNA complex formation was analyzed by nondenaturing PAGE (6%) and autoradiography, and analyzed quantitatively by AlphaImaging.

RNA strand annealing assays

These assays were adapted from Gabus et al [32]. Briefly, 0.015 pmol of ^{32}P-labeled RNA was incubated, with or without target RNA, in the presence of recombinant FMRP, FMRP I304N, FMRP ΔKHT, or FXR1P [30] protein at 1 : 1, 2 : 1, or 4 : 1 (protein:RNA), or BSA (B) at 4 : 1 molar ratio. Assays were performed in 10 μL containing 20 mM Tris-HCl (pH 7.0), 0.1 mM $MgCl_2$, 30 mM NaCl, 10 μM $ZnCl_2$, 5 mM DTT, 5% Superase-In at 37°C for 10 min. The reaction was stopped by chilling on ice. After adding 0.5 volume of stop mix (20% glycerol, 20 mM EDTA pH 8.0, 0.2%

SDS, 0.4 mg/mL yeast tRNA, and 0.25% bromophenol blue) to chase FMRP, the samples were analyzed by nondenaturing 10% PAGE in TBE buffer. The gels were dried and the radioactive RNA complexes were visualized by autoradiography, and analyzed quantitatively by PhosphorImaging.

DNA constructs, cell culture, and luciferase assays

The psiSTRIKE and psiCHECK vectors were obtained from Promega. Mouse embryonic *Fmr1* KO (STEK TSV-40, 3T3A, 3T6A, or TpBSVE) [24, 30, 33], *Fxr1* KO (KETS) (to be described elsewhere) and wild-type (Naïves or NIH/3T3) fibroblasts were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator under 5% CO_2 at 37°C. Expression of FMRP, FXR1P, and actin proteins was verified by Western blot using antimAb1C3 [34], mAb2FX [31], and anti-actin AC-40 (Sigma) antibodies, respectively. Cells grown in 12-well plates to 70% confluency were transfected with 0.8 μg of the psiCHECK reporter vector and either 0.0005–0.5 μg of the psiSTRIKE construct using Lipofectamine 2000 (Invitrogen) or 100–400 nM of ss antisense Rluc siRNA (asRluc) or 100 nM of Rluc siRNA duplex (siRluc) using Oligofectamine (Invitrogen). Twenty-four hours later, cells were harvested, lysates were prepared, and Rluc and Fluc activities were measured successively using the Dual-Luciferase Reporter Assay System (Promega).

RESULTS

FMRP acts as an acceptor of miRNAs derived from Dicer cleavage

Dicer activity has been reported to be associated with FMRP in EBV-transformed human B cells [29], suggesting that Dicer and FMRP are part of a complex in vivo. Since FMRP is likely acting downstream of Dicer in the RNA silencing pathway, we asked whether FMRP can act as a miRNA acceptor protein for the ribonuclease Dicer. To test this hypothesis, the ^{32}P-labeled miRNA precursor pre-miR-31 was incubated in the presence of recombinant Dicer, which bound (Figure 1(a), left panel) and cleaved pre-miR-31 into a $\sim$ 21-nt RNA product (Figure 1(a), center panel). When this ^{32}P-labeled RNA derived from Dicer cleavage was gel-purified and incubated with increasing amounts of recombinant FMRP, a gel mobility shift was observed, compatible with the formation of an FMRP-RNA complex (Figure 1(a), right panel).

The relative ability of FMRP to interact with RNA species involved in RNA silencing was further quantified by EMSA. Initial analyses showed that recombinant FMRP associates with the human immunodeficiency virus transactivating response DNA [32] and RNA (I Plante and P Provost, unpublished data) structure, the latter of which closely resembles that of a pre-miRNA. We observed that FMRP preferentially recognized mature miRNA (Figure 1(b), right), as monitored by the concomitant decrease in unbound RNA levels. Under these conditions, FMRP only weakly bound a perfect siRNA (Figure 1(b), left), or an imperfect miRNA:miRNA* (Figure 1(b), center) duplex. These results confirm the ability of FMRP to bind to the effector mature miRNA.

FMRP assembles miRNAs and ss siRNAs on target RNAs in vitro

Given the ability of FMRP to interact with several hundreds of mRNAs [35, 36] and its recently described nucleic acid chaperone properties [32], we asked if FMRP could facilitate the assembly of miRNAs on target RNAs. To this end, single miRNA strands were incubated with a specific target RNA in the presence or absence of recombinant FMRP. Formation of imperfectly paired miRNA:target RNA complexes was visualized by nondenaturing PAGE after FMRP removal. We observed FMRP-mediated assembly of cel-let-7 (Figure 2(a), left panel) and hsa-miR-196a-1 (Figure 2(a), right panel) miRNAs on their specific 60-nt RNA targets. No annealing was observed in the presence of bovine serum albumin (BSA) (Figure 2(a), 7th lane of each panel) or when the cel-let-7 target *lin-41* was swapped with that of hsa-miR-196a-1 (*HOXB8*) (Figure 2(a), last lane of each panel). Whether this activity could be extended to siRNAs was tested using siRNAs directed against Dicer and coactosin-like protein (CLP) mRNAs. In both cases, assembly of single siRNA strands on their 100-nt RNA targets was observed (Figure 2(b)), but not when the targets were interchanged (Figure 2(b), last lane of each panel). Used as hybridization control reactions, temperature-mediated annealing of ss siRNAs to their targets was superior to that of miRNAs, as expected considering the increased stability of perfectly complementary ss siRNA:target RNA versus imperfect miRNA:target RNA duplexes. In contrast, FMRP favored annealing of miRNAs to their targets, more than that of ss siRNAs (compare Figure 2(a) versus Figure 2(b)), thereby illustrating its preference for imperfect, but natural miRNA:target RNA complex formation.

FMRP mediates miRNA annealing to specific RNA targets through its KH domains

To get further insights into the miRNA annealing properties of FMRP, we assessed the activity of the biologically relevant FMRP I304N mutant and the deleted ΔKHT FMRP version. A single substitution (I304N) in the *Fmr1* gene product FMRP was found in a fragile X patient suffering of a severe form of fragile X syndrome [37]. When the recombinant FMRP I304N mutant protein was tested in RNA strand annealing assays, it exhibited properties comparable to the wild-type protein (Figure 3(a)). On the other hand, deletion of the KH domains markedly compromised its miRNA annealing properties (Figure 3(b)). Since the KH domains present in RNA-binding proteins are known to mediate binding to ss nucleic acids, as reviewed recently [38], these results support the requirement of the ssRNA-binding KH domains for FMRP-mediated miRNA annealing to specific RNA targets.

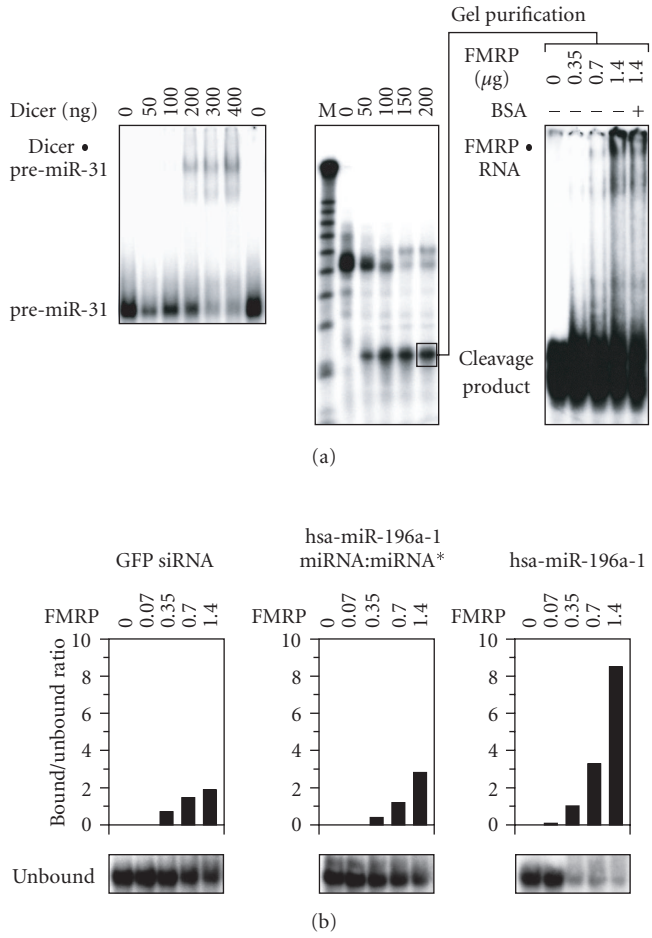


Figure 1: FMRP can act as an acceptor of miRNA derived from Dicer cleavage of pre-miRNA. (a) Recombinant FMRP interacts with Dicer RNA cleavage products. ^{32}P-labeled pre-miR-31 was incubated in the absence or presence of recombinant Dicer, without (left) or with (center) $MgCl_2$. The samples were analyzed by nondenaturing (left) or denaturing (center) PAGE and autoradiography. RNA derived from Dicer cleavage was gel-purified and incubated with increasing amounts of recombinant FMRP (0.35–1.4 μg), without or with BSA (20 μg), prior to EMSA analysis (right). M indicates a 10-nt RNA size marker. (b) FMRP preferentially interacts with miRNAs. ^{32}P-labeled green fluorescent protein (GFP) siRNA (left), hsa-miR-196a-1 miRNA:miRNA* duplex (center), or miRNA (right) was incubated in the absence or presence of increasing amounts of recombinant FMRP. The samples were analyzed by EMSA and autoradiography, and analyzed quantitatively by PhosphorImaging. Bound RNA was expressed as a bound to unbound ratio.

FMRP is required for efficient RNA silencing in mammalian cells

In order to assess whether the activity displayed by FMRP in vitro is important for RNA silencing in vivo, we set up a reporter-based cellular assay using cultured mouse embryonic fibroblasts established from *Fmr1* KO [24] or isogenic wild-type mice. In these assays, a dual reporter gene construct encoding *Renilla* (*Rluc*) and *Firefly* (*Fluc*) luciferase was cotransfected with a construct encoding an shRNA directed against Rluc. We used a second *Fluc* reporter that, in addition to correct for cell-to-cell variability, allowed us to examine the function of FMRP pertaining to small RNA-mediated RNA silencing. This was essential considering that FMRP can act as a negative regulator of translation [22–24].

Immunoblot analyses of *Fmr1* KO cell protein extracts confirmed the absence of FMRP (Figure 4(a), right lane), whereas FMRP was detected in wild-type cells (Figure 4(a), left lane). Expression of Rluc shRNA, which had no effect on interferon regulatory factor-1 (IRF-1) protein levels (see Supplementary Data, Figure S1), induced a dose-dependent decrease in Rluc activity in wild-type cells (Figure 4(b)). The efficiency of Rluc shRNA to induce RNA silencing was significantly impaired in *Fmr1* KO cells, as compared to wild-type cells.

The observed variations in RNA silencing responses might be a consequence of looking at different cell line clones which, apart from being deficient in FMRP, may have different properties with respect to transfection, nucleic acid release, or amount of available Exportin 5 [39] or RISC [40].

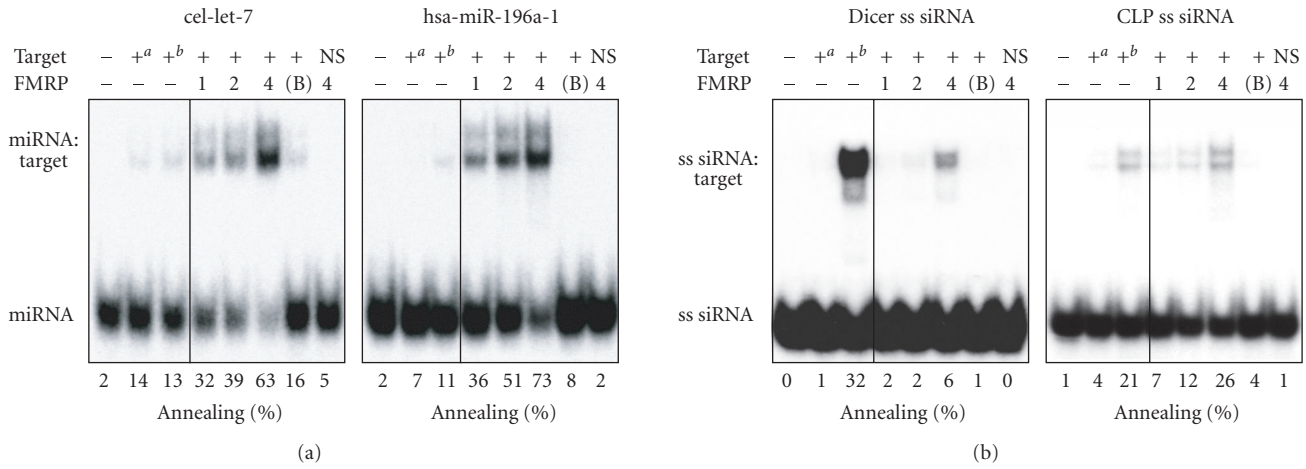


Figure 2: FMRP preferentially facilitates natural miRNA:target RNA complex formation. FMRP mediates miRNA (a) and siRNA (b) annealing to specific RNA targets. (a) ^{32}P-labeled cel-let-7 (left) or hsa-miR-196a-1 (right) was incubated with a specific *lin-41* or *HOXB8* RNA target, respectively, in the absence or presence of increasing amounts of FMRP, or BSA at 4 : 1 (protein:RNA) molar ratio. (b) The RNA strand annealing assays using a single ^{32}P-labeled strand of Dicer (left) or CLP (right) siRNA duplex incubated with a specific or nonspecific RNA target were performed as in (a). Control hybridization was conducted at 37°C for 10 min^a or 65°C for 30 min^b. RNA complexes were analyzed by nondenaturing 10% PAGE and autoradiography. B, BSA; NS, nonspecific target.

A recent study reported that the efficiency of the RNAi pathway may differ between cell types [41]. In order to confirm our data and to avoid possible misinterpretation due to cell line-specific RNA silencing responses, we have tested three additional *Fmr1* KO cell lines (STEK 3T3A, STEK 3T6A, and STEK TpBSVE) and one additional control wild-type cell line (NIH/3T3). The absence or presence of FMRP in these cell lines was confirmed by immunoblot analysis. As shown in Figure 4(c), impairment of shRNA-induced Rluc silencing was observed in all *Fmr1* KO cell lines tested, as compared to FMRP-expressing control cell lines. Thus, the observed defect of the RNA silencing pathway in *Fmr1* KO cell lines is not specific to a particular cell line clone, thereby confirming the importance of FMRP for efficient RNA silencing in mammalian cells.

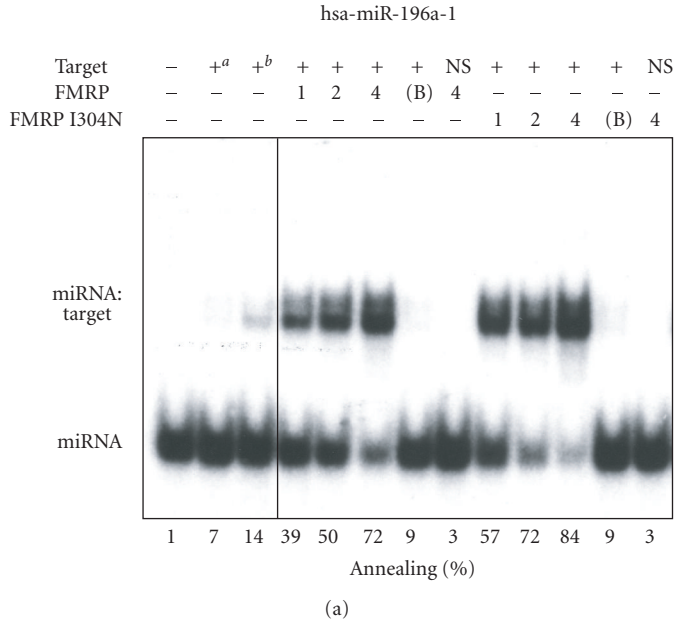
FMRP is essential for ss siRNA-induced RNA silencing

In order to get further insights into the role of FMRP in RNA silencing, we compared in *Fmr1* KO and wild-type cells the efficiency of two other RNA silencers (siRNA duplex and ss antisense siRNA) that are known to enter the endogenous machinery at levels downstream to that of shRNAs or pre-miRNAs. Duplex siRNA-induced silencing was efficient in wild-type cells, with a > 90% inhibition of Rluc activity at 100 nM, but was defective in *Fmr1* KO cells ($P < .001$) (Figure 5). These results suggest that the defect associated with the absence of FMRP is situated at or downstream of the siRNA duplex entry level into the RNAi pathway.

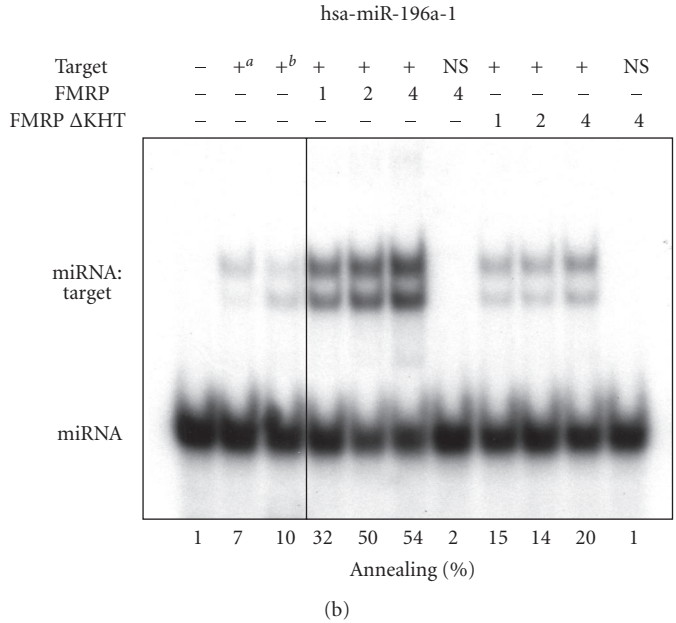
To characterize this perturbation further, we induced silencing of the Rluc reporter gene by using ss antisense siRNA, which may enter the RNA silencing machinery downstream of the strand selection/separation step(s). In wild-type cells, the extent of Rluc silencing induced by ss antisense Rluc siRNA reached ∼ 35% at the highest concentration (Figure 5), which was less pronounced than that achieved with the corresponding duplex, as previously reported [42, 43]. However, cells lacking FMRP were incompetent at mediating Rluc silencing induced by ss antisense Rluc siRNA ($P < .05$). These data imply the involvement of FMRP in a distal ss siRNP effector complex in mammalian cells.

The properties of FMRP are shared by its paralog FXR1P

In contrast to the single *dFMR1* gene in *Drosophila*, three paralogs, namely, FMRP and the fragile X-related proteins FXR1P and FXR2P, are expressed in mammalian cells [31]. In order to examine if the properties of FMRP are unique or representative of this gene family, we studied the function of FXR1P. As illustrated in Figures 6(a) and 6(b), the miRNA and ss siRNA annealing properties of FXR1P are similar to those exhibited by FMRP in vitro (compare with Figures 2(a) and 2(b)). We then studied the importance of FXR1P in RNA silencing in vivo using *Fxr1* KO cells. Immunoblot analyses confirmed the absence of FXR1P in these cells (Figure 6(c), compare right lane versus left lane). As *Fmr1* KO cells, *Fxr1* KO cells also exhibited, although to a lesser extent, a compromised RNA silencing response relative to wild-type cells (Figure 6(d)). These results suggest a common, possibly complementary, role for members of the fragile X protein family in RNA silencing in mammals.



(a)



(b)

Figure 3: FMRP mediates miRNA annealing to specific RNA targets through its KH domains. RNA strand annealing properties of the FMRP I304N (a) and ΔKHT (b) mutants. (a) ^{32}P-labeled hsa-miR-196a-1 miRNA was incubated with a specific *HOXB8* RNA target in the absence or presence of FMRP or FMRP I304N or with BSA at 4 : 1 (protein:RNA) molar ratio. (b) The RNA strand annealing assays using the FMRP ΔKHT mutant were performed as in (a). Control hybridization was conducted at 37°C for 10 min^a or 65°C for 30 min^b. RNA complexes were analyzed by nondenaturing 10% PAGE and autoradiography. B, BSA; NS, nonspecific *lin-41* RNA target.

DISCUSSION

A cooperation between Dicer and FMRP, in which FMRP would act as an acceptor of miRNAs derived from Dicer processing of pre-miRNAs, is consistent with a concerted action in regulating mRNA expression. Independent groups have shown that target cleavage directed by ss siRNA is supported in extracts of HeLa cells [42, 43], from which a human RISC containing ss siRNA was characterized [42]. The presence of a single siRNA strand in mRNA-cleaving RNP complexes, previously found to contain dFMR1 [27, 28], has also been demonstrated in *Drosophila* S2 cells [44, 45]. Although the

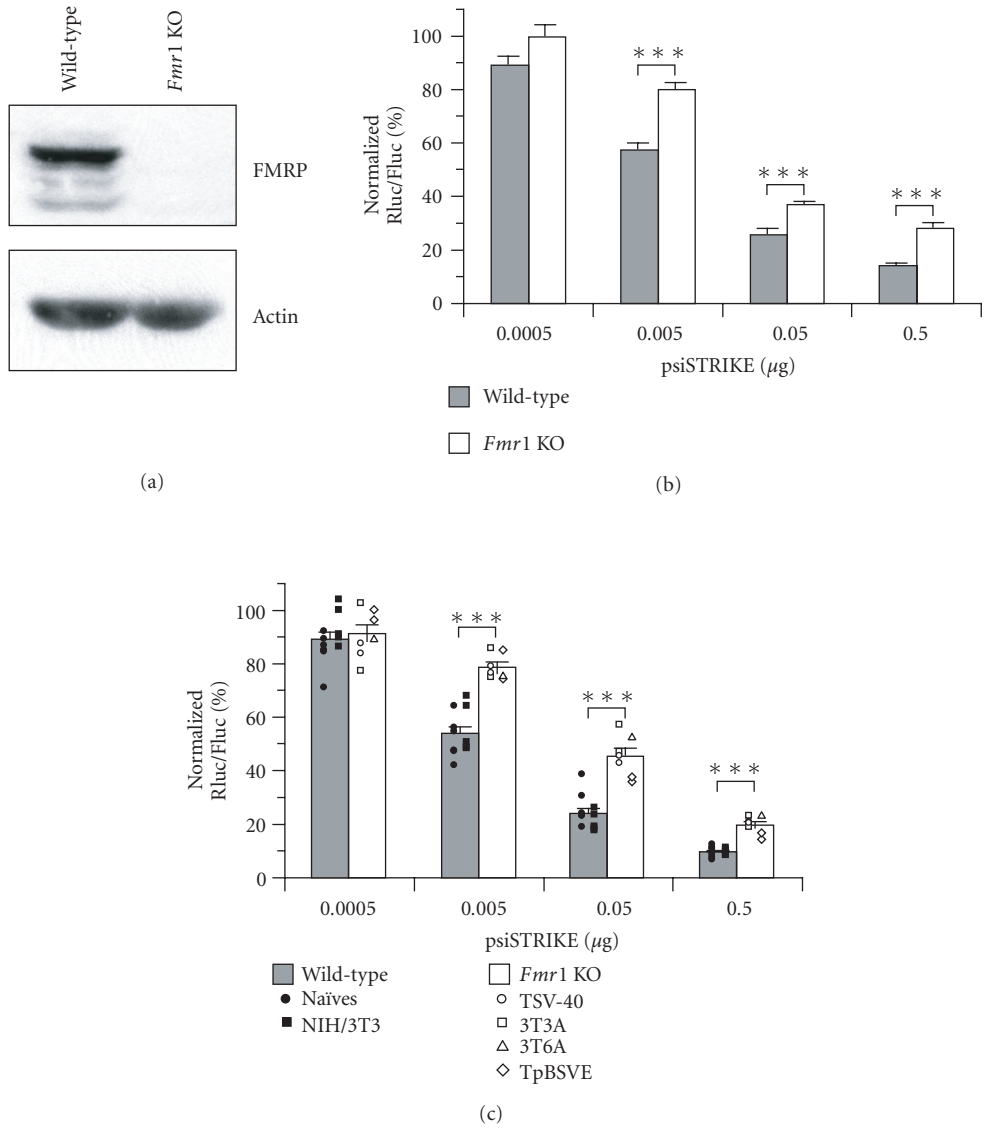


Figure 4: FMRP is required for efficient RNA silencing in mammalian cells. (a) Expression of FMRP in wild-type and *Fmr1* KO (TSV-40) cells was verified by immunoblot analysis, in parallel with actin. (b) *Fmr1* KO (TSV-40) and wild-type (Naïves) cells were cotransfected with psiSTRIKE encoding Rluc shRNA and psiCHECK reporter construct ($n = 6$). (c) *Fmr1* KO cell lines STEK TSV-40 ($n = 2$), STEK 3T3A ($n = 2$), STEK 3T6A ($n = 1$), and STEK TpBSVE ($n = 2$), and wild-type Naïves ($n = 6$) and NIH/3T3 ($n = 5$) cell lines were cotransfected as in (b). Results of Rluc activity were normalized with Fluc activity and expressed as a percentage of Rluc activity obtained with an shRNA directed against a sequence deleted in the Rluc reporter mRNA. Results were expressed as mean ± SEM and analyzed by analysis of variance followed by unpaired Student's *t* test. $^{***}P < .001$.

human and fly RNA silencing machineries differ mechanistically and in terms of mediating translational repression or mRNA cleavage, they may share two features in common: an ssRNA-containing effector RNP complex and a member of the fragile X protein family.

Recent findings indicate that siRNA-bound Dicer-2 assembles into RISC in *Drosophila* [44–46]. Although the assembly of miRNA-bound Dicer into human RISC remains to be demonstrated, the observations that (i) the product of human Dicer remains associated with the enzyme [7], that (ii) Dicer interacts directly with the human RISC component Ago2 [47], and that (iii) FMRP forms an RNP complex with Ago2 and miRNAs in mammalian cells [29] are compatible with this scenario. This assembly scheme may determine the nature of the RNA species to be loaded into RNPs and confer specificity to the general RNA binding and chaperone properties of FMRP operating in miRNA-mediated RNA silencing. Although a recent study demonstrating the presence of

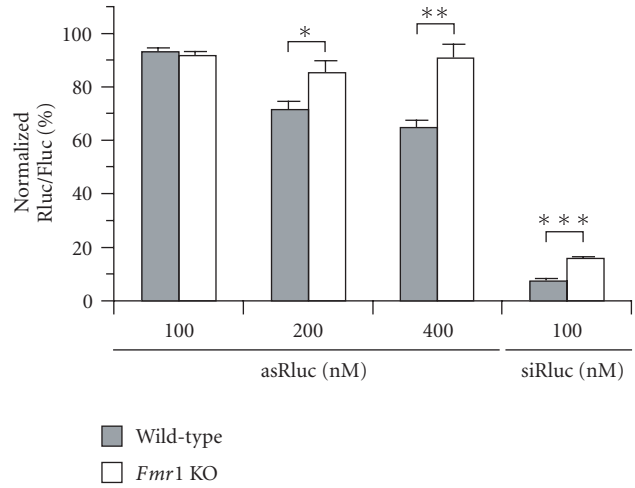


Figure 5: FMRP is required for ss siRNA-induced RNA silencing. *Fmr1* KO (TSV-40) and wild-type (Naïves) cells were cotransfected with ss antisense Rluc siRNA (asRluc) or Rluc siRNA duplex (siRluc) and psiCHECK reporter construct ($n = 6$). Results were expressed as mean ± SEM and analyzed as described in the legend of Figure 4. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

Dicer preassembled in a human RISC that can cleave target RNA by utilization of a pre-miRNA Dicer substrate offers a slightly different perspective [48].

When assessing cellular responses to silencing RNAs, we found that FMRP was required for optimal RNA silencing in mammalian cells, as previously reported in *Drosophila* [27]. Rluc silencing mediated by Rluc shRNA is likely executed by an ss siRNP through a reaction in which the strand annealing properties of FMRP are solicited. This is in agreement with the fact that FMRP is unable to exchange strands of perfectly paired siRNA duplexes to target RNAs in vitro (see Supplementary Data, Figure S2) and recent evidences suggesting the involvement of an ss siRNP in target RNA cleavage mediated by human RISC [48]. Considering the transposition of our in vitro annealing assays, using synthetic RNAs and recombinant proteins, to RNA silencing in vivo, the caveat has to be taken into account that the presence of other protein or nucleic acid components as well as postranslational modifications may influence the role and function of FMRP.

The relative importance of fragile X proteins in RNA silencing observed in our study may have been underestimated given that expression of FXR1P and FMRP may have contributed to alleviate and compensate for their respective functional loss in *Fmr1* and *Fxr1* KO cells. A relatively higher expression levels of FMRP and Dicer in *Fxr1* KO fibroblasts was observed (I Plante and P Provost, unpublished data). This may be related either to a compensatory mechanism, in the case of the former, or a dysfunctional miRNA-based regulation of their expression. The fact that FMRP and FXR1P exhibit similar activities, conjugated with the differential tissue expression patterns of the fragile X proteins observed in mammals, suggests that RNP complexes of various composition may be functionally redundant.

A fragile X patient was found to carry a single substitution (I304N) in the *Fmr1* gene product FMRP. We observed that recombinant FMRP I304N mutant protein exhibited miRNA strand annealing properties comparable to the wild-type protein. In contrast, the ss nucleic acid binding KH domains appear to be required for the miRNA annealing activity of FMRP. However, neither of these two mutants, that is, FMRP ΔKHT [30] and FMRP I304N [49], cosedimented with polyribosomes, thereby dissociating the polyribosomal association and miRNA annealing activity of FMRP. These observations suggest that the phenotype observed in fragile X patients may be related either to the loss of its chaperone activity [32] or the mislocalization of a still active mutant protein, as demonstrated for the FMRP I304N mutant [49].

The hypothesis that the subset of mRNAs bound by FMRP may be subjected to miRNA regulation, as proposed by Jin et al [50], is attractive. FMRP may recognize its target mRNAs through recognition of the G quartet structures [51, 52] and/or via the kissing complex motifs [53]. The silencing state of the bound mRNA might then be determined by a specific regulatory miRNA. The specificity of miRNA-based translational regulation may thus reside in both the sequence of the miRNA as well as the recognition of selected mRNA targets by FMRP. In turn, the presence and importance of FMRP in effector miRNP complexes may explain why FMRP is found associated with so many mRNAs. It will be interesting to see what proportion of the several hundreds of mRNAs bound by FMRP [54, 55] are experimentally validated and physiologically relevant miRNA targets.

The biological significance of pre-miRNA binding by FMRP (I Plante and P Provost, unpublished data), an event that would occur upstream of Dicer, is unclear. However, it is relevant to note that FMRP contains a nuclear localization

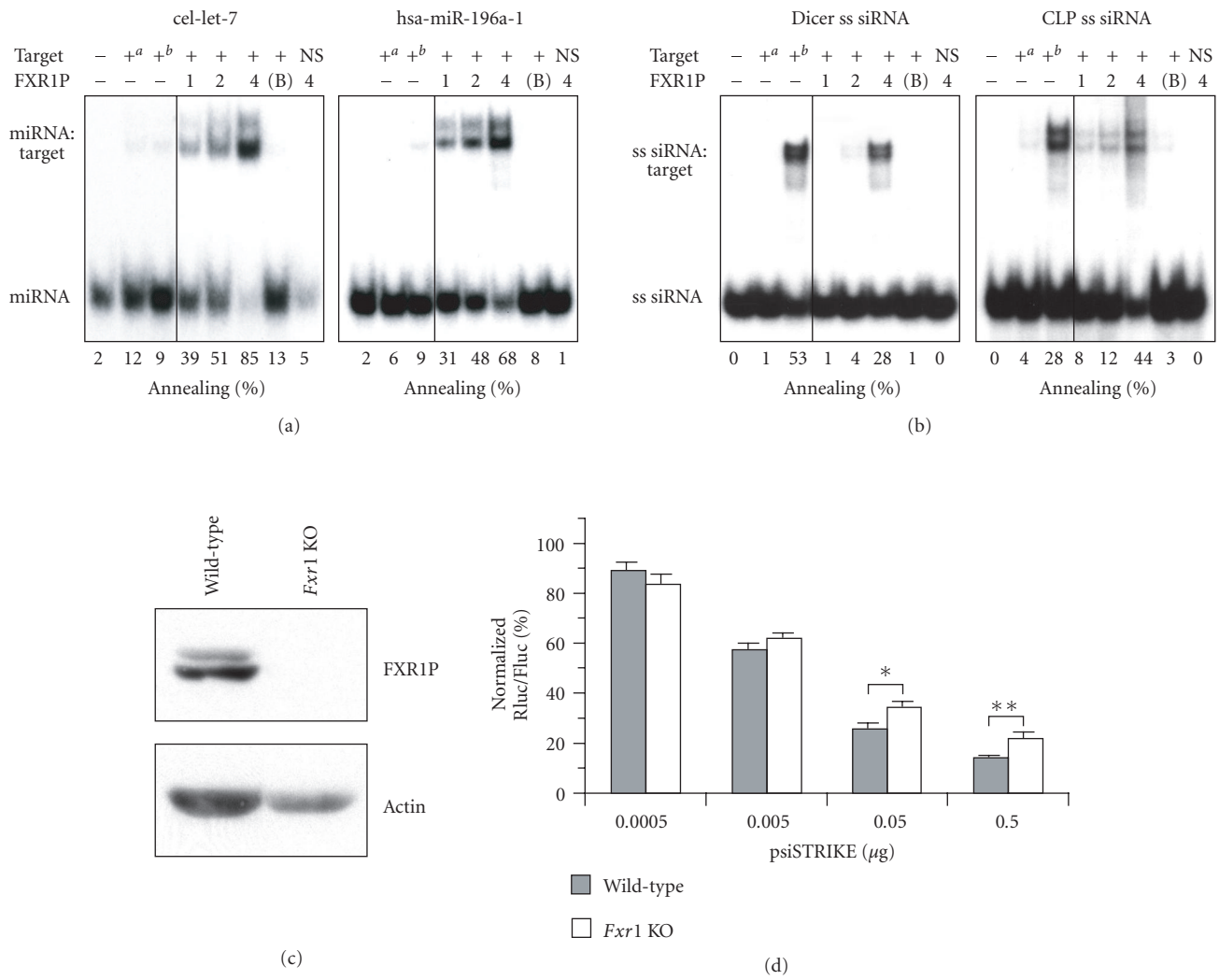


Figure 6: The paralog FXR1P shares the RNA strand annealing properties of FMRP. (a)-(b) RNA strand annealing assays with FXR1P were performed and analyzed as described in the legend of Figure 2. (c) Expression of FXR1P in wild-type and *Fxr1* KO cells was verified by immunoblot analysis, in parallel with actin. (D) *Fxr1* KO and wild-type (Naïves) cells were cotransfected with psiSTRIKE encoding Rluc shRNA and psiCHECK reporter construct ($n = 6$). Results were expressed as mean ± SEM and analyzed as described in the legend of Figure 4. $^{*}P < .05$; $^{**}P < .01$.

signal (NLS) as well as a nuclear export signal (NES) [56]. These sequences appear to be functional, as FMRP was subsequently characterized as a nucleocytoplasmic shuttling protein [57]. Together, these observations raise the possibility that FMRP may play a role in nuclear export of pre-miRNA. The issues as to whether FMRP can be found in processing bodies mediating translation repression, as suggested by trapping of mRNAs by FMRP into cytoplasmic granules [24], or accompanies miRNP complexes involved in other cellular processes requiring mRNA repression, such as transfer of mRNAs from the cell body to dendrites of neurons [58], will also need to be addressed.

Several findings suggest the possible presence of Dicer in FMRP-containing complexes present in the translation machinery: (i) fractionation of competent RISC with ribosomes [10]; (ii) the demonstration that Dicer is part of the human RISC [48]; (iii) the documented existence of polyribosomal miRNAs [59, 60]; and (iv) transcripts engaged in translation regulation are sensitive to RNAi [61]. Our findings provide yet another functional link between the initiation and effector steps of RNA silencing.

FMRP may function in RNA silencing as a downstream effector of Dicer and miRNA assembler on target mRNAs. FMRP is particularly abundant in the brain due to its high expression in neurons [34, 62], and the extent of neuronal miRNA-mediated mRNA regulation, as deduced by the number and diversity of miRNAs cloned from mammalian neurons [60], appears to be important. A suboptimal miRNA

assembly on their natural mRNA targets, resulting from the lack of FMRP expression, may contribute to the molecular basis for the fragile X syndrome, thereby suggesting a causal link between dysfunction of the RNA silencing machinery and a human disease.

ABBREVIATIONS

EMSA	electrophoretic mobility shift assay
FMRP	fragile X mental retardation protein
KH	K-homology
KO	knockout
miRNA	microRNA
pre-miRNA	miRNA precursor
pri-miRNA	primary miRNA
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase	ribonuclease
RNP	ribonucleoprotein
sh	short hairpin
siRNA	small interfering RNA
ss	single-stranded
FXR1P	fragile X-related protein 1

ACKNOWLEDGMENTS

We thank Stéphane Richard for critical reading of a previous version of the manuscript and Guy Rousseau for helpful discussions. E. W. Khandjian is indebted to Gideon Dreyfuss for the gift of *Fxr1* KO mouse embryos. L. Davidovic was supported by a postdoctoral fellowship from the FRAXA Research Foundation and L.-A. Gobiel by a master studentship from the Natural Sciences and Engineering Research Council of Canada (NSERC). P Provost is a New Investigator of the Canadian Institutes of Health Research (CIHR). This work was supported by Grant NDG-70190 from CIHR-Institute of Genetics and CIHR-Institute of Neuroscience, Mental Health and Addiction (to P. Provost and E. W. Khandjian), Grant IGI-44012 from CIHR (to E W Khandjian), and Grant EOP-64706 from Health Canada/CIHR (to P. Provost). P. Provost is the author of correspondence.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 16806, Pages 1–7
DOI 10.1155/JBB/2006/16806

Research Article

Hypothesis: A Role for Fragile X Mental Retardation Protein in Mediating and Relieving MicroRNA-Guided Translational Repression?

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Received 8 April 2006; Accepted 2 May 2006

MicroRNA (miRNA)-guided messenger RNA (mRNA) translational repression is believed to be mediated by effector miRNA-containing ribonucleoprotein (miRNP) complexes harboring fragile X mental retardation protein (FMRP). Recent studies documented the nucleic acid chaperone properties of FMRP and characterized its role and importance in RNA silencing in mammalian cells. We propose a model in which FMRP could facilitate miRNA assembly on target mRNAs in a process involving recognition of G quartet structures. Functioning within a duplex miRNP, FMRP may also mediate mRNA targeting through a strand exchange mechanism, in which the miRNA* of the duplex is swapped for the mRNA. Furthermore, FMRP may contribute to the relief of miRNA-guided mRNA repression through a reverse strand exchange reaction, possibly initiated by a specific cellular signal, that would liberate the mRNA for translation. Suboptimal utilization of miRNAs may thus account for some of the molecular defects in patients with the fragile X syndrome.

THE miRNA-GUIDED RNA SILENCING MACHINERY AND DISEASES

MicroRNAs (miRNAs) are small ~21- to 24-nucleotide (nt) RNAs that mediate messenger RNA (mRNA) translational repression through recognition of specific partially complementary binding sites usually located in the 3′ nontranslated region (NTR). They are generated by successive processing of stem-loop structured primary miRNAs (pri-miRNAs) and miRNA precursors (pre-miRNAs) by the ribonucleases (RNases) III Drosha [1] and Dicer [2–4], respectively, as reviewed in this issue by Ouellet et al [5]. Encoded by 1–5% of the genome in eukaryotes, miRNAs may regulate more than 30% of the genes in humans [6, 7]!

Unveiling the complexity of the process, recent studies have identified new protein components involved in miRNA-guided RNA silencing, such as DiGeorge syndrome critical region 8 (DGCR8) [8–11], transactivating response (TAR) RNA-binding protein (TRBP) [12–15], and fragile X mental retardation protein (FMRP) [16, 17]. Intriguingly, these accessory proteins, which are required for optimal functioning of the pathway, are related to specific human diseases. Thus, in addition to the emerging causal links between defective miRNAs and human diseases, as reviewed in Ouellet et al [5] and Perron et al [18], some genetic disorders might also be related to malfunctioning of the machinery involved in miRNA biogenesis and function.

DGCR8 was identified in Drosha immunoprecipitates and found to be required for optimal pri-miRNA processing [10]. Both acting within the Microprocessor complex [10], DGCR8 has been proposed to guide Drosha in substrate recognition [11]. Clinically, patients carrying a common monoallelic deletion affecting the DGCR8 gene display phenotypes defined as the DiGeorge syndrome, with manifestations such as congenital heart defects, characteristic facial appearance, immunodeficiency, and behavioral problems [19, 20]. The possibility that the DiGeorge syndrome is caused by a perturbed Drosha function and/or pri-miRNA processing is attractive, but remains to be established.

As for TRBP, it was identified by proteomic analysis of Dicer immunoprecipitates and reported to assist Dicer function within a pre-miRNA processing complex [12, 13]. Initially identified in 1991, TRBP was characterized as a cellular factor acting in synergy with the viral Tat protein in the

transactivation of the long terminal repeat of human immunodeficiency virus type 1 (HIV-1), leading to viral gene transcription [21]. TRBP may thus play a dual role in HIV-1 pathogenesis and RNA silencing, as recently discussed [22].

Two independent groups simultaneously reported a possible relationship between the *Drosophila* FMRP ortholog (dFMR1) and RNA interference (RNAi). dFMR1 was found to be associated with the effector RNA-induced silencing complex (RISC) as well as miRNAs in *Drosophila* S2 cells [16, 17]. In mammalian cells, FMRP was reported to be part of a ribonucleoprotein (RNP) complex with Argonaute 2 (Ago2) and miRNAs [23]. These findings suggest a possible link between the loss of FMRP function in miRNA-guided RNA silencing and the fragile X syndrome.

FMRP AS A REGULATOR OF TRANSLATION

In humans, the *FMR1* (fragile mental retardation 1) gene, which spans ~38 kb in the q27.3 region located at the tip of the X chromosome long arm, encodes an mRNA of ~3.9 kb composed of a ~0.2 kb 5′NTR, a 1.9 kb coding region, and a 1.8 kb 3′NTR [24]. Loss of the *FMR1* gene product FMRP is the etiologic factor of the fragile X syndrome, the most frequent cause of inherited mental retardation [25, 26]. It affects about 1 in 4000 males, who will develop in almost all cases moderate to severe mental retardation (IQ ≤ 50), and about 1 in 7000 females, who present in general a milder mental handicap [24].

FMRP has been detected in practically every tissue in humans and rodents, with high levels in the brain, testes, esophagus, lung, and kidney [27]. The ability of FMRP to bind RNA, which was first suggested by the presence of K-homology (KH) domains and an RGG box found in various RNA-binding proteins, was later confirmed experimentally [28, 29]. FMRP is associated with translating polyribosomes in neuronal cells [30, 31] and acts, at high levels, as a negative regulator of translation in vitro and in vivo [32–34].

Experimental studies have documented the involvement of FMRP in translational control. When preincubated with mRNAs, FMRP leads to inhibition of translational inhibition both in vitro in the rabbit reticulocyte lysate system and in vivo after microinjection in the *Xenopus* oocytes [32, 33]. In *Drosophila*, the dFMR1 protein was reported to down-regulate expression of the *futsch* protein [35]. Expression of high levels of FMRP results in repression of reporter genes in transfected cultured mammalian cells [34]. Although FMRP has been shown to interact with mRNAs and to inhibit translation, its exact role and function are unclear.

More recently, using the recombinant protein, Gabus et al [36] have shown that FMRP possesses nucleic acid chaperone properties, shedding new light on the principal mechanism by which FMRP could regulate gene expression. They observed that FMRP could either anneal or transfer DNA strands of TAR that are perfectly complementary in vitro. Examining these activities in a hammerhead ribozyme model system, the authors noted that FMRP enhanced ribozyme cleavage of an RNA substrate. The KH motifs and RGG box were found to be important for optimal chaperone activity

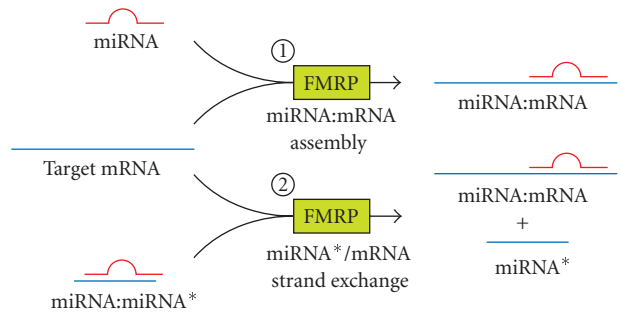

Figure 1: FMRP can assemble and exchange strands of RNAs involved in miRNA-guided RNA silencing. Recombinant FMRP can (1) assemble an miRNA to its specific mRNA target, or (2) use an miRNA:miRNA* duplex for mRNA targeting through an RNA strand exchange reaction, which releases the miRNA*.

[36]. These findings suggest that FMRP may regulate translation by acting on the structural status of mRNAs.

Are these nucleic acid chaperone properties of FMRP compatible with, and relevant to, a specific cellular context or regulatory process, such as miRNA-guided RNA silencing?

A ROLE FOR FMRP IN miRNA-GUIDED RNA SILENCING?

In *Drosophila* S2 cells, mRNA-cleaving RNP complexes have been found to contain dFMR1 [16, 17] as well as a single siRNA strand [37, 38]. Independent groups have shown that target cleavage directed by single-stranded (ss) siRNAs is supported in extracts of HeLa cells [39, 40], from which a human RISC containing ss siRNAs was characterized [40]. In mammalian cells, FMRP was reported to be part of an miRNA-containing RNP (miRNP) complex containing Ago2 [23]. Thus, although the mammalian and fly RNA-silencing machineries differ substantially, they may share two features in common: an RNP complex containing an ssRNA species and a member of the fragile X family of proteins.

How miRNAs and ss siRNAs are used by the effector RNP complexes for recognition and targeting of regulatable mRNAs remains poorly understood. However, rather than being the result of a passive hybridization reaction, formation of an miRNA:mRNA or ss siRNA:mRNA transition complex is more likely to be facilitated by a component of the miRNP or siRNP complexes. Capable of promoting RNA folding and hybridization, FMRP represents the most interesting and valuable protein candidate.

FMRP FACILITATES miRNA ASSEMBLY ON TARGET mRNA

We verified this possibility and showed that human FMRP can act as an miRNA acceptor protein for Dicer and facilitate assembly of miRNAs on specific target RNA sequences (see Figures 1 and 2) [41]. In these assays, FMRP exhibited a preference for assembling imperfectly paired miRNA:mRNA duplexes, which is the most prevalent situation encountered in mammals. The miRNA assembler property of FMRP was

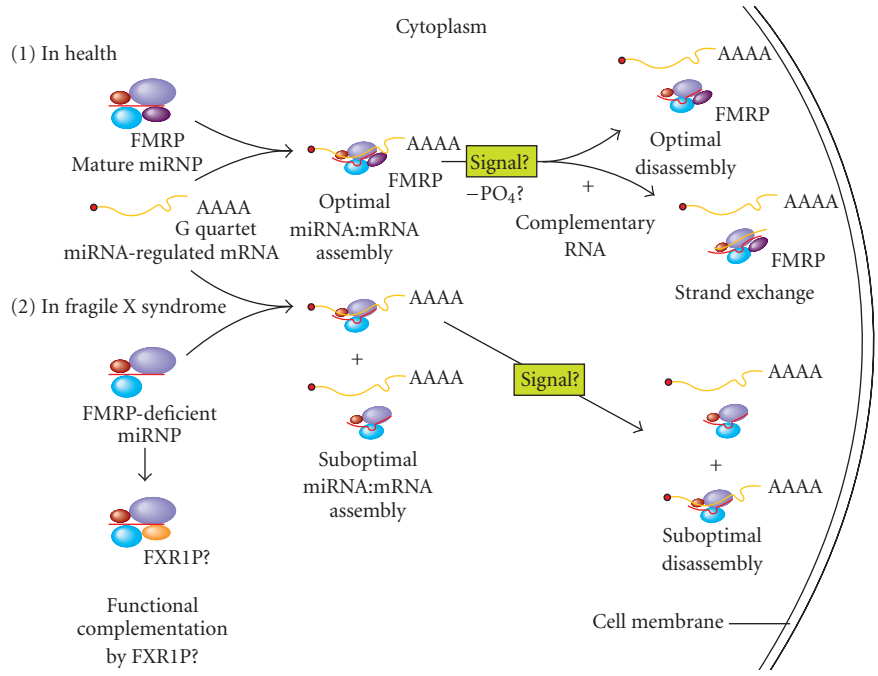

Figure 2: Proposed roles and functional regulation of FMRP in miRNA-guided RNA silencing. (1) mRNA targeting by mature miRNPs may involve the concerted assembly of the miRNAs to their binding sites with the recognition of G quartet structures in specific mRNAs by FMRP (in purple). Acting within miRNPs, FMRP may play a key role by relieving translational repression through disassembly of the miRNA:mRNA complexes. mRNAs repressed by miRNPs could thus be reutilized and translated into proteins. This process may be triggered by a cellular signal, possibly a dephosphorylation event. A variant of that phenomenon would have a complementary RNA, like the miRNA*, involved in a strand exchange reaction making the mRNA available for translation, while recycling the miRNP into a duplex miRNA-containing RNP. (2) In the absence of FMRP, miRNA assembly on target mRNAs may be suboptimal. Moreover, FMRP-deficient miRNPs may be less responsive to signal-induced miRNA:mRNA disassembly, resulting in a suboptimal dissociation from the target mRNAs. In certain tissues where it is expressed, the FXR1P paralog (in orange) may functionally complement, and possibly compensate for, the absence or loss of FMRP expression.

abrogated upon deletion of its RNA-binding KH domains [41]. In line with these findings, reporter gene silencing assays supported the involvement of FMRP in an ss siRNA-containing RNP (siRNP) effector complex and revealed its requirement for optimal RNAi in cultured mammalian cells [41]. These data suggest that FMRP may function as the miRNA assembler in RNA silencing.

FMRP CAN EXCHANGE miRNA*/mRNA STRANDS

Recently, native gel electrophoresis unveiled different RNP complexes containing siRNA duplexes in *Drosophila* [37, 38], leading the authors to propose a siRNP assembly scheme compatible with conversion of double-stranded (ds) siRNP into ss siRNP. mRNA cleavage activity could only be correlated with the latter complex [37]. siRNA-induced mRNA cleavage and miRNA-guided translational repression may be mediated by different RNP complexes and differ mechanistically. If ss siRNPs induce mRNA cleavage, what about the complexes that mediate inhibition of translation? Do they contain ssRNA or dsRNA, such as miRNA duplexes? In fact, duplex RNA-containing RNPs may be more than precursors of ssRNA-containing RNPs. They may be functionally important in RNA silencing. Whether they are involved in miRNA-guided translational repression has not been addressed so far.

However, this would require resolution of the "three-strand" enigma, posed by targeting of an ssRNA by dsRNA species. In that context, formation of a specific miRNA:mRNA transition complex from a miRNA:miRNA* duplex and its target RNA would appear as obligatory. Interestingly, human FMRP can do just that! We observed that FMRP can accept and utilize miRNA:miRNA* duplexes, generated upon Dicer processing of pre-miRNAs, to favor miRNA:mRNA complex formation through an miRNA*/mRNA strand exchange reaction [41], as illustrated in Figure 1. Together, these observations suggest that FMRP could facilitate mRNA targeting by acting (i) as an miRNA assembler in ssRNA-containing RNPs, and (ii) as an miRNA*/mRNA strand exchanger in duplex RNA-containing RNP effector complexes.

IS FMRP INVOLVED IN RELIEVING mRNA REPRESSION?

Reversibility is a fundamental difference between siRNP-mediated mRNA cleavage or miRNP-guided translational repression. If mRNA structural and functional integrity is preserved, silenced mRNAs could be translated again,

whereas degraded mRNAs could not. In *Drosophila*, the RISC mediates cleavage of the mRNA target, and is presumably regenerated, allowing for repeated cycles of mRNA inactivation and amplification of the phenomenon [43]. In contrast to the most prevalent situation in flies and plants, mammalian mRNAs are mainly and primarily subjected to miRNP-induced translation repression prior to their relocation to specific cytoplasmic foci, referred to as processing (P-bodies) [44, 45] or GW182-containing bodies (GW-bodies) [46], where mRNA degradation occurs. This sequence of events is supported by a recent study revealing that mRNAs containing partial miRNA complementary sites are eventually targeted for degradation in vivo, as exemplified by the lin-41 mRNA:let-7 miRNA tandem [47].

Do P-bodies represent the unique and ultimate destination of miRNA-repressed cellular mRNAs? Can these mRNAs be prevented from reaching their final destination? Is there a defined structural or functional point of no return, ahead of which mRNAs could be redirected towards the translation machinery? Most interestingly, can mRNAs escape and return "safe" from P-bodies and be used again for protein synthesis? In these cases, the involvement of a protein having the ability to shuttle between cellular compartments may be expected.

miRNPs may be perceived as translational "locks" and contribute to preserve mRNA structural and functional integrity until it needs to be translated again. Relief of the miRNA-guided translational repression may thus represent a posttranscriptional control of gene expression relevant to situations where specific proteins need to be expressed within minutes under specific circumstances, such as conditions of cellular stress. The exact mechanism and duration of miRNA-induced repression of a given mRNA remain unclear at this point. As well, no information is currently available as to how long the mRNA can be stored when complexed with miRNPs. However, if miRNA repression of mRNAs can be relieved, it would likely involve a coordinated and regulated disassembly of miRNA:mRNA complexes, a process probably executed by a component of miRNPs, such as FMRP.

FMRP MAY ACT AS A MOLECULAR SWITCH IN miRNA-GUIDED RNA SILENCING

How could miRNA repression be relieved? It may be initiated upon destabilization of miRNP binding to the repressed mRNA, induced by unknown cellular signals or factors, leading to dissociation of the miRNA:mRNA complex (see Figure 2). Whether differences in strand complementarity of the complexes can contribute or suffice to drive this reaction backward requires further investigation.

Alternatively, the RNA strand exchange properties of FMRP may participate to the relieving of miRNA repression, in a reaction possibly involving the miRNA*, whose fate and function remain obscur. A recent study by Matranga et al [48] showed that the siRNA passenger strand of the duplex is cleaved by Ago2 in *Drosophila* embryo lysates. However, the authors showed that passenger-strand cleavage is not important for the incorporation of miRNAs that derive from mismatched duplexes [48]. These observations raise the following question: do miRNAs* play a significant role in RNA silencing?

The possibility that the miRNA* could be used by FMRP like a spare RNA for the mRNA to be relieved from the miRNA repression, as illustrated in Figure 2, is plausible and attractive. Characterized as a chaperone [36], FMRP could bind to one or more nucleic acid molecules and promote the formation of the most stable structure, upon which its continued binding is no longer required to maintain it [36, 49, 50]. The results of our previous study [41] suggest that the miRNA:mRNA complex is more stable than the miRNA:miRNA* duplex. We hypothesize that unknown cellular signals or factors may assist FMRP in executing a reverse strand exchange by lowering the required thermodynamic threshold. The miRNA* may further facilitate that process by providing an appropriate sequence-specific RNA template. The reaction would liberate the mRNA for translation and lead to reconstitution of a duplex miRNP, theoretically available for subsequent rounds of mRNA regulation events.

A ROLE FOR FMRP DEPHOSPHORYLATION IN RNA SILENCING?

FMRP may function as a molecular device regulating mRNA translation by allowing optimal miRNA assembly or disassembly in response to specific cellular signals or factors. Posttranslational modifications, such as phosphorylation/dephosphorylation events, may regulate FMRP function in RNA silencing, allowing it, for example, to switch between the forward (on) and reverse (off) modes of RNA strand exchange. *Drosophila* FMR1 is phosphorylated by casein kinase II at serine 406 (Ser406), which is highly conserved among fragile X family members from several species [51]. This residue is located in close proximity to the RGG box and corresponds to Ser499 [52] or Ser500 [51] (depending on amino acid numbering) in human FMRP, which was also found to be phosphorylated in vivo. Phosphorylation upregulated dFMR1 oligomerization, thereby enhancing the RNA-binding properties of the protein [51]. In contrast to the *Drosophila* ortholog, the phosphorylation status of mammalian FMRP did not influence its association with specific mRNAs in vivo [52]. The authors, however, found that unphosphorylated FMRP is associated with actively translating polyribosomes, while a fraction of phosphorylated FMRP is associated with apparently stalled polyribosomes [52]. These data suggest that the release of FMRP-induced translational suppression may involve a dephosphorylation signal [52].

FMRP MAY BE A KEY DETERMINANT OF miRNA:mRNA SPECIFICITY

The presence and importance of FMRP in effector miRNP complexes may explain why FMRP is found associated with several hundreds of different mRNAs [53]. It will be interesting to see what proportion of these mRNAs bound by FMRP [53, 54] is experimentally validated and physiologically

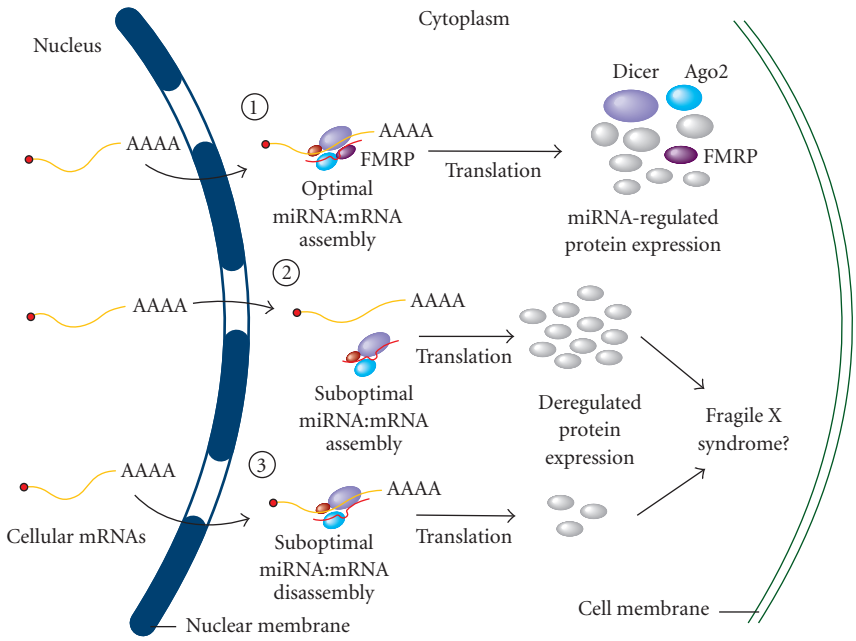

FIGURE 3: A potential role for suboptimal miRNA:mRNA assembly and/or disassembly in the etiology of the fragile X syndrome. (1) miRNA assembly on target mRNAs may be optimal when facilitated by FMRP, which may also be required for optimal relief of miRNA-guided translational repression. (2) In the absence of FMRP, suboptimal miRNA assembly on target mRNAs may lead to overexpression of specific proteins. (3) Whereas suboptimal disassembly of FMRP-deficient miRNPs from target mRNAs may result in a prolonged inhibition of translation. The resulting misbalanced expression of miRNA-regulated genes may alter downstream cellular processes and metabolic pathways (not depicted in this figure) and lead to the development of the fragile X syndrome.

relevant miRNA targets. Using a bioinformatic approach, John et al [42] observed a strong enrichment of predicted targets in mRNAs associated with FMRP in mammals. FMRP may thus be a key determinant of miRNA:mRNA specificity. This is consistent with the hypothesis that miRNAs act as sequence-specific adaptors in the interaction of RNPs with translationally regulated mRNAs [42]. This interaction may be dictated, on the one hand, by the nucleotide sequence of the miRNA and, on the other hand, by the presence of G quartet structures [55] and/or kissing complex motifs [56] in the mRNA. Therefore, it may not be a coincidence that the *FMR1* gene is highly conserved among vertebrates [27], where mRNAs are primarily subjected to miRNP-induced translation repression.

A MOLECULAR BASIS OF THE FRAGILE X SYNDROME?

The biochemical properties of FMRP make it a prime candidate for a role in mediating and relieving miRNA-guided translational repression. We hypothesize that the absence of FMRP expression may result in suboptimal miRNA assembly on, and/or disassembly from, their natural mRNA targets, leading to a perturbed protein expression profile (see Figure 3). This may be expected given the requirement of FMRP for efficient small RNA-guided gene regulation [41].

The FMRP paralog fragile X-related protein 1 (FXR1P) exhibited miRNA annealing and strand exchange properties similar to FMRP [41]. These data suggest a conserved function for fragile X proteins in RNA silencing and open the possibility that FXR1P may partly complement or compensate for the absence or loss of FMRP expression (see Figure 2).

The next challenge will be to ascertain the ability of FMRP to assemble and exchange miRNA strands in a cellular context. In fact, several additional issues need to be addressed in order to validate the proposed hypothesis. For instance, are these properties of FMRP preserved within miRNPs in vivo? What are the cellular proteins and/or cofactors assisting FMRP function in vivo? Is FMRP able to relieve miRNA-guided mRNA repression? If so, what is the mechanistic of that process, that is, the sequence of events, nature of the components involved and/or signal(s) required? When and where in the cell do these events take place? Elucidation of the exact role and function of FMRP in miRNA-guided gene regulation may hold key to determining the molecular basis of the fragile X syndrome and establishing a causal link between dysfunction of the RNA-silencing machinery and a human genetic disease.

ACKNOWLEDGMENTS

We are grateful to Edward Khandjian for encouragement and support, Gilles Chabot for graphic design, and members of the Provost laboratory for stimulating discussions. P. Provost is a New Investigator of the Canadian Institutes of Health Research (CIHR). This work was supported by Grant NDG-70190 from CIHR-Institute of Genetics and/or CIHR-Institute of Neuroscience, Mental Health, and Addiction and Grant EOP-64706 from Health Canada/CIHR. P. Provost is the author for correspondence.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 73104, Pages 1–8
DOI 10.1155/JBB/2006/73104

Review Article

Noncoding RNAs in Cancer Medicine

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Received 20 January 2006; Accepted 3 April 2006

Several signalling proteins involved in cell growth and differentiation represent attractive candidate targets for cancer diagnosis and/or therapy since they can act as oncogenes. Because of their high specificity and low immunogeneicity, using artificial small noncoding RNA (ncRNAs) as therapeutics has recently become a highly promising and rapidly expanding field of interest. Indeed, ncRNAs may either interfere with RNA transcription, stability, translation or directly hamper the function of the targets by binding to their surface. The recent finding that the expression of several genes is under the control of small single-stranded regulatory RNAs, including miRNAs, makes these genes as appropriate targets for ncRNA gene silencing. Furthermore, another class of small ncRNA, aptamers, act as high-affinity ligands and potential antagonists of disease-associated proteins. We will review here the recent and innovative methods that have been developed and the possible applications of ncRNAs as inhibitors or tracers in cancer medicine.

INTRODUCTION

The accumulation of multiple genetic alterations that affect the activity and/or expression of key proteins confers the proliferative and invasive characteristics of growth to cancer cells. Chromosomal deletions, rearrangements, and gene mutations are selected during cancer progression because these defect(s) lead to altered protein signalling networks and generate a survival advantage for the cancer cell [1].

The sequencing of the human genome, coupled to the availability of novel techniques as the high throughput screens and microarrays analysis, in less than a decade, has led to a vast accumulation of information about genes that are aberrantly regulated in cancers and has generated the realistic hope of identifying, at the molecular level, the fundamental processes that cause transformation from normal cell growth to malignancy.

The implications of this knowledge are profound because a detailed understanding of the complex interactions that occur at the genetic and protein levels provides attractive targets for rationally designing new drugs for new prevention and treatment approaches. Indeed, a major challenge of cancer research studies is to distinguish individuals at high risk of developing cancer thus to develop improved strategies for earlier diagnosis and more effective treatment with minimal side effects.

In the recent few years the increasing understanding on the function of small noncoding RNAs (ncRNAs) has as well generated a great enthusiasm because these molecules may provide an obvious potential use as powerful new tools in cancer medicine. Under the definition of ncRNAs falls a broad range of regulatory RNA molecules, such as ribozymes, antisense, interfering small RNAs or aptamers, that are either naturally found in several cell types or are artificially designed to target gene expression or protein function (Figure 1). The advantage of these biomolecules over other biochemical or chemical substances employed up to now include high potency and specificity for the target, use of in vitro techniques for their production, that considerably reduce production costs as well as the need for animal testing and that markedly increase specificities and quality assurance in diagnostic and therapeutic applications.

In this review, we will examine recent work in the possible applications of artificial small ncRNAs as versatile biomolecules to identify and validate cancer targets and as inhibitors or tracers in cancer medicine. The advantages and drawbacks of the competing methodologies will be discussed here.

A HETEROGENEOUS FAMILY OF RNA-BASED TOOLS

Small noncoding RNAs elicit at least four distinct types of responses that trigger specific gene inactivation, including

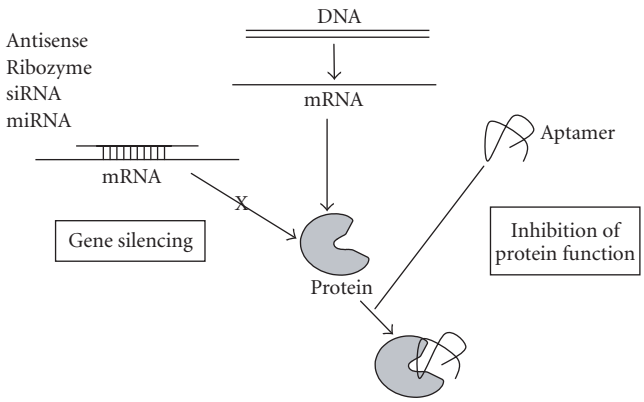

Figure 1: Schematic representation of the mode of action of aptamers compared to other ncRNAs. Antisense, ribozymes, siRNAs, miRNAs recognise the target nucleic acid by complementary base pairing and, by activating an intracellular molecular machinery, impair the expression of the corresponding protein. Aptamers act by directly binding the target without interfering with its expression.

destruction of homologous mRNA, inhibition of translation, de novo methylation of genomic regions that can block transcription of target genes, and chromosomal rearrangement.

Among small RNAs, the microRNAs (miRNAs) are 21-base-long RNA molecules that regulate gene expression. In mammalians, miRNAs act by imperfectly base-pairing predominantly within the 3′ untranslated region of target messenger RNAs and inhibiting protein translation [2]. Because of their importance in the regulation of gene expression miRNAs have been implicated in the modulation of several physiological and pathological cellular processes.

In RNA silencing mediated by siRNAs the sequence-specific gene inhibition is initiated by small RNA duplexes that cause the destruction of complementary target messenger RNA.

Aptamers are single-stranded oligonucleotides whose inhibiting function relies on a completely different mechanism with respect to miRNA and siRNA. These molecules are generated by an in vitro evolutionary selection-amplification scheme, named SELEX (systematic evolution of ligands by exponential enrichment) [3, 4]. Because they bind their target molecules at high affinity and specificity, displaying dissociation constants in the low picomolar to low nanomolar range, aptamers are considered as attractive therapeutic agents that rival antibodies.

As shown in Figure 2, the starting point for the generation of an aptamer is the synthesis of a nucleic acid library (RNA, DNA, or modified RNA) of large sequence complexity followed by the selection for oligonucleotides able to bind with high affinity and specificity to a target molecule. Randomisation of a synthetic sequence stretch from 22 up to 100 nucleotides in length has been used to create an enormous diversity of possible sequences (4^N different molecules) which in consequence generate a vast array of different conformations with different binding properties. The SELEX method includes the following steps: (i) incubating the library with the target molecule under conditions favourable for binding; (ii) partitioning: molecules that, under the conditions employed, adopt conformations that permit binding to a specific target are then partitioned from other sequences; (iii) dissociating the nucleic acid-protein complexes; and (iv) amplifying of the nucleic acids pool to generate a library of reduced complexity enriched in sequences that bind to the target. This library will be then used as starting pool for the next round of selection. After reiterating these steps for a variable number of cycles, the resulting oligonucleotides are subjected to DNA sequencing. The sequences corresponding to the initially variable region of the library are screened for conserved sequences and structural elements indicative of potential binding sites and subsequently tested for their ability to bind specifically to the target molecule. This selection scheme works since single-stranded nucleic acids fold up into unique 3D shapes in a similar manner to proteins, each structure being unique and dictated by the sequence of the nucleic acid.

By starting with 10^{15} random DNA sequences (thus, to a first approximation, 10^{15} specific shapes), it is possible to select (through 10–15 rounds of selection-amplification) specific binding reagents for virtually each targeted human protein.

UNDERSTANDING THE MOLECULAR MECHANISMS OF CANCER

Determining gene function

Determining by reverse genetics the function and the biological relevance of a given protein for a particular cancer type is a critical step to validate the most promising molecular targets for drug development. The possible strategies that are usually used to understand the function of a specific gene in a cell are either based on techniques that impair the expression of the candidate target gene or rely on the use of products that act by specifically interfering or inhibiting the function but not the expression of the final product. In both cases the resulting phenotype turns out as a powerful source of information on the function of the target protein (Figure 1).

(a) *Gene silencing*: the generation of null mutants by homologous recombination of a given gene in a cell or in an entire organism has been extensively used to create models of several human diseases, including cancer. Using this technique (named gene knockout), in which the gene of interest is irreversibly disrupted and the synthesis of the encoded products abolished, allowed to make an incredible and rapid progress in our understanding of the function of several oncogenes and tumor-suppressor genes. However, triggering gene silencing by homozygous gene ablation is laborious and expensive and thus rather inappropriate for a large-scale screening. An alternative strategy has been recently developed to determine the roles of particular genes in cancer that is based on the use of ncRNAs for gene silencing. The RNA interference (RNAi) has proven to be a precious approach that permits loss-of-function phenotypic screens in mammalian somatic cells or in whole animals at high specificity.

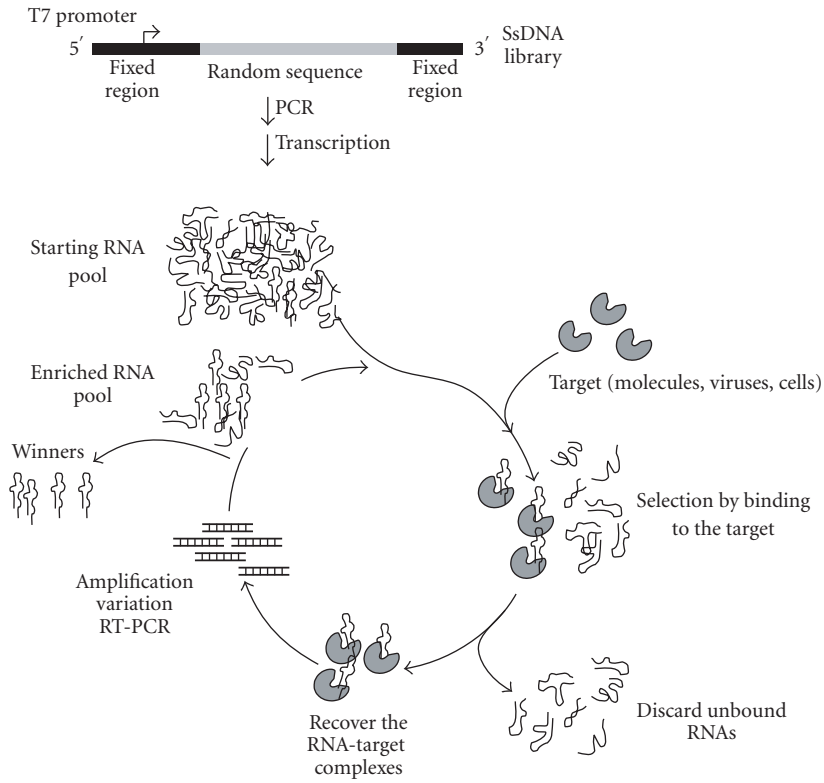

Figure 2: Schematic representation of the SELEX process. The single-stranded (ss) DNA library is amplified by polymerase chain reaction (PCR) in order to generate the double-stranded DNA pool that will be transcribed by T7 RNA polymerase. The pool of RNA molecules with different conformations will be used for the selection process (see text for details).

Indeed, in the last few years the sequencing of the entire human genome coupled to advancements in high-throughput oligonucleotide synthesis and better prediction of active sequences is allowing the design of RNAi constructs against virtually any transcript. Furthermore, in contrast to the knockout approach, the RNA interference-based strategies achieve loss of function phenotypes without the loss of genomic information of the targeted gene (recently reviewed in 5). This leaves the possibility to restore the exact expression of the endogenous gene once the RNAi vector is silenced or removed.

(b) *Functional inhibitors*: a major drawback to gain information on the function of a given protein by impairing its expression is that proteins and enzymes involved in crucial functions, such as cell growth and differentiation, frequently act in concert with various partners thus forming large stable complexes that dictate its function in the cell environment. Therefore, depleting a single key protein from the cell will change, or even disrupt, at the same time one or more of these multiprotein complexes. As a consequence, the resulting phenotype will be produced by the simultaneous impairment of several protein functions and the understanding stays frequently ambiguous. Furthermore, silencing a gene gives no information about which region of the putative target protein is important for its function.

To overcome these disadvantages, additional approaches have been developed that enable to interfere with a given protein function. Indeed, using monoclonal antibodies, peptides, and small molecules to directly target the protein in a drug-like manner has the advantage to interfere with the protein activity without depleting the protein itself and thus with low destabilisation of the proteomic status of the cell. During the past decade, as excellent alternatives to these inhibitors, the RNA-based aptamers have proven to be highly selective ligands and efficient inhibitors of a wide variety of proteins implicated in cancer. Aptamers have a larger surface area as compared to small interfering compounds thus presenting more points of contact with the target protein. Furthermore, these molecules have been shown either to inhibit their target by competitive mechanisms or to interfere with its conformation by noncompetitive mechanisms [6]. The ability to select aptamers directed against purified soluble targets has recently incredibly progressed thanks to the automation of part of the in vitro selection processes so that several targets can be isolated in parallel strongly reducing the time required for the selection. Aptamers for protein targets of biomedical interest have been reported and many of them are actually under clinical trials for cancer treatment (Table 1). In particular, Food and Drug Administration has recently approved one aptamer developed by Eyetech (Macugen^{TM}) that inhibits the human vascular endothelial growth factor 165 (VEGF165), for the treatment of age-related macular degeneration [7]. Since an obvious

Table 1: Therapeutic aptamers in cancer treatment.

Aptamer	Aptamer activity		The rapeutic application
	in vitro	in vivo	
Macugen	Inhibition of VEGF165	Inhibition of the VEGF-induced vascular permeability	Approved by FDA for treatment of age-related macular degeneration
ProMune	Agonist for toll-like receptor 9 (TLR 9)	Activate the immune system through TLR 9 against cancer	Phase 2: melanoma Phase 1: renal cell carcinoma; non- Hodgkin's lymphoma; cutaneous T-cell lymphoma, non-small-cell lung cancer
Agro 100	Binding to nucleolin	Antiproliferative activity in a broad array of tumor cell types; enhancement of chemotherapeutic agents effects	Phase 1 Phase 2 launched in 2005 for advanced solid malignancies
HYB2055	Agonist for TLR 9	Antitumor activity in nude mouse xenografts with colon, breast, lung cancer, and glioma cell lines	Phase 2 for advanced solid malignancies
VaxImmune adjuvant	Agonist for TLR 9	Elicits a powerful immune response against infectious disease and cancers	Phase 2 for several different cancer indications

potential therapeutic use for aptamers to VEGF is in cancer, this aptamer was tested in a mouse model of nephroblastoma [8]. Renal histopathology revealed an 84% reduction in tumor weight in the aptamer-treated kidneys compared to the controls. Furthermore, lung metastases were seen in 20% of the aptamer-treated mice compared to 60% of control animals. This same aptamer was also tested in a murine model of neuroblastoma, where it resulted in 53% reduction in tumor growth compared to control [9]. Another aptamer that inhibits receptor tyrosine kinase activation by binding to the corresponding ligand is the aptamer against platelet-derived growth factor (PDGF). It has been successfully used in vivo in animal models of cancer due to its high specificity in the fact that it suppresses PDGF B-chain (PDGF-BB) but not the epidermal- or fibroblast-growth-factor-2-induced proliferation [10]. Furthermore, the SELEX methodology has been used to identify high-affinity 2'-aminopyrimidine RNA ligands to the potent angiogenic factor, the basic fibroblast growth factor (bFGF). In cell culture, these aptamers inhibited bFGF binding to both low-affinity sites and high-affinity sites on FGF receptor-1 [11].

Even though several aptamers that inhibit receptor tyrosine kinases by binding to their soluble ligands have now been selected, targeting the receptor itself that is a large insoluble glycosylated protein has only been recently addressed [12]. To this aim we developed a general protocol of differential whole-cell SELEX to target cell surface bound proteins in their natural physiological environment. We have evolved aptamers able to inhibit an active mutant of the receptor tyrosine kinase, Ret, by targeting its extracellular domain in which such activating mutation is located. By this method, the selection procedure was performed by using as target the RET^{C634Y} mutant expressed on PC12 cells. A library of 2′-fluoro pyrimidine RNAs was incubated with parental PC12 cells to remove aptamers that bind nonspecifically to the cell surface. To select for aptamers that specifically bound the mutant receptor, the supernatant was incubated with PC12–RET^{C634Y} cells. Unbound sequences were washed off, the whole process reiterated 16 times, and the bound winning sequences cloned. The resulting aptamers did not bind to a recombinant EC C634Y RET fragment highlighting the strength of the whole-cell approach. Among the selected aptamers, the best inhibitor (D4) binds specifically to the Ret receptor tyrosine kinase and blocks its downstream signalling effects on cell differentiation and transformation [12]. The results suggest that the differential whole-cell SELEX approach will be useful in the isolation of other lead therapeutic compounds and diagnostic cell-surface markers. Aptamers that have high affinity and specificity for tissues have also been produced, demonstrating that complex targets, including tumour tissue, are compatible with the SELEX process. "Tissue SELEX" methodology could be favourable when the precise molecular target is unknown but the target is, for example, a specific type of cells. A fluorescence based SELEX-procedure was applied against transformed endothelial cells as a complex target to detect microvessels of rat experimental glioma, a fatal brain tumour which is highly vascularized. A secondary selection scheme, named deconvolution-SELEX, was carried out to facilitate the isolation of ligands for components of interest within the targeted mixture. Other examples of proteins that have been reported as targets for development of aptamers as promising therapeutics include the tenascin-C, the Ras binding domain of Raf-1, the prostate specific membrane antigen (PMSA), the protein kinase CβII, the epidermal growth factor receptor-3 (ErbB3/HER3), and the CXCL10 chemokine [13].

Identifying "cancer genes"

Two paradigmatic and elegant recent papers well demonstrate the power of siRNAs in identifying new cancer targets

involved in cell survival. Apoptosis-based anticancer therapies are designed to achieve tumor eradication through the use of death-inducing molecules. Because of its specific toxicity against transformed cells, the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors are judged to be amongst the most promising apoptosis-based antitumor targets. To better understand the molecular mechanisms of TRAIL-induced apoptosis, Aza-Blanc et al [14] carried out a large RNAi-based screen to identify genes that modulate TRAIL-induced apoptosis. They used an siRNA library to individually target 510 human genes (corresponding to 380 predicted kinases and 130 other predicted proteins) transfected in the cervical carcinoma derived HeLa cell line. By using an assay that could detect both sensitization and resistance to TRAIL-induced apoptosis the authors identified a variety of genes that modulate both positively and negatively TRAIL activity. Furthermore, besides genes that encode well-characterised mediators of TRAIL, including the DR4 receptor for TRAIL and the caspase 8 or known modulators of apoptosis (Myc and JNK3), in the RNAi screen they detected and functionally characterised two previously unknown genes, which were found to modulate TRAIL-induced apoptosis. One of the genes, DOBI (Downstream of Bid), is required for progression of the apoptotic signal through the intrinsic mitochondrial cell death pathway indicating that it may function to mediate cytochrome *c* release induced by BID cleavage, the other, named MIRSA (Mina53-related suppressor of apoptosis), is a gene that acts to prevent TRAIL-induced death.

Changes in the expression and/or activity of kinases and phosphatases, key proteins of the cellular signalling pathways, are the most frequent molecular causes of cancer progression. MacKeigan et al [15] reported a large-scale RNAi approach to identify kinases and phosphatases that regulate cell survival and apoptosis. Authors transfected in HeLa cells two large siRNA library sets against human kinase and phosphatase, containing two siRNAs for each target (corresponding to 650 kinases and to 222 phosphatases). By the data obtained they predicted that 11% of kinases and 32% of phosphatases are constituted of genes whose expression is critical for cell survival. In addition to those for previously known "survival kinases" (Akt2, KD2, SGK, PKCdelta) they identified several genes with still unknown function, the silencing of two of which resulted in a strong increase in apoptosis. They also identified phosphatases that act as tumor suppressors to sensitize or promote apoptosis. Silencing of these "cell death phosphatases" resulted in marked cell protection to chemotherapeutic-induced cell death. Furthermore, the authors developed a further screen in the presence of low doses of chemotherapeutics by which they identified kinases whose silencing increases the rate of apoptosis. The kinases identified are thus promising targets for silencing in order to sensitize the cancer cell to low doses of chemotherapeutic agents, thus reducing unwanted side effects in chemotherapy.

On the other hand, as alternative to transfection, siRNAs can be also expressed within the cells as a short hairpin RNA (shRNA) that is then processed by the cell machinery to produce the small interfering double strand RNA. To facilitate the use of RNAi as a genetic tool in mammals, Paddison et al [16] and Berns et al [17] used a shRNA retrovirus-based library approach to develop strategies that allow a high throughput RNAi-based screen of mammalian genes. Berns et al screened a library targeting around 8.000 human genes for those that affect the function of the tumour suppressor, p53. Genes were subsequently identified by microarray detection of the shRNA sequence [17]. Paddison and colleagues developed a shRNA library targeting around 10.000 human and more than 5.000 mouse genes. To facilitate the use in virtually any cell types, their shRNA expression library was constructed in a vector that permits moving the shRNA encoding inserts to different vectors by bacterial mating and designed to function for both genetic selections and screens. Indeed, for facilitating the screening, in addition to the selection pressure both groups adopted a unique DNA "bar-code" sequence present in the vector which can be identified using microarrays containing oligonucleotides corresponding to the bar-code sequences.

The generation of large siRNA libraries has been further improved by using, instead of shRNAs that are transcribed by the RNA polymerase III, pri-miRNA based large transcripts that permit to generate siRNAs driven by the RNA polymerase II promoters that can be thus tightly regulated both in culture and in vivo, in animal models [18, 19].

Two recent reports [20, 21] address the use of large viral-based RNAi libraries to identify novel potential tumor-suppressor genes thus further underscoring the power of RNAi screening to understand the molecular mechanisms of neoplastic transformation.

Kolfschoten et al [20] used a shRNA retrovirus-based library [17] in combination with in vitro neoplastic transformation assays to screen for novel tumor-suppressor genes. To this aim they used a Ras-dependent transformation model of genetically modified human primary BJ fibroblast cells expressing the catalytic subunit of telomerase (hTERT), and SV40 small T antigen (ST) in combination with the inhibition of the expression of p53 and $p16^{INK4A}$. In this cell line the expression of the oncogenic H-Ras^{V12} is sufficient to confer anchorage-independence for survival and proliferation [22]. By this approach the authors identified few genes whose silencing substitutes for the activity of the oncogenic Ras one of which, the homeodomain transcription factor PITX1, was not previously implicated as possible tumor suppressor allowing anchorage-independent growth of fibroblasts. The authors showed that PITX1 regulates the Ras pathway and thereby tumorigenesis. The mechanism appears to involve constitutive activation of the Ras signalling pathways at the level of GTP loading onto Ras itself. Indeed, PITX1 directly controls the expression of RASAL1, one of the negative regulators of Ras belonging to the GTPase-activating protein (GAP) family. The evidence that PITX1 is a tumor suppressor was supported by the strong correlation between the low PITX1 levels present in colon cancer cell lines and wt Ras expression and the low PITX1 expression levels in prostate and bladder tumor tissues compared with normal tissues.

To identify genes that suppress oncogenic transformation, Wetsbrook et al [21] used genetically modified human

epithelial mammary cells that have been immortalized by the expression of hTERT and SV40 large T antigen and also naturally express high levels of Myc (TLM-HMECs). Forced expression of an active mutant of PI3K confers to these cells the ability to grow in an anchorage-independence manner [23]. Based on the assumption that inactivation of a single tumor-suppressor gene may be sufficient to shift TLM-HMECs cells into a frankly transformed phenotype that can be selected for, the authors used a shRNA retrovirus-based library to infect TLM-HMECs [16]. In the screen they identified several potential suppressors of epithelial cell transformation genes that represent potential tumor suppressors. Most of them were associated with genes known to be involved in key intracellular signalling pathways including Ras, PI3K, and TGF-β signalling. In addition, they provided evidence that one of these candidates, the transcriptional repressor REST/NRSF, plays a previously unknown role in tumor suppression. REST encodes a transcriptional factor involved in repressing neuronal genes in non-neuronal cells. Even though the mechanism by which REST silencing releases the transformed state should be still elucidated, it likely involves regulation of PI3K. Indeed, impaired REST function promoted epithelial cell transformation, enhanced the intensity and duration of PI3K activity, eventually acting via the transcriptional control of neurotrophins.

Despite the expanding potentiality of siRNAs the key challenges for their development for gene silencing is largely dependent on the improvement of siRNA specificity. Not every sequence works, and a success rate of about one of three should be expected. In addition, although the effects are generally thought to be highly sequence specific, one potential concern in using siRNAs for phenotypic screens is that the observed effects could be due to inhibition of either the intended target or of an off-target mRNA [24]. Indeed, siRNAs are not perfectly selective and results should be confirmed with an independent siRNA targeting of the same transcript to understand whether or not some of the effects see result from an off-target transcript. Generation of libraries of multiple siRNAs for each gene has been therefore the most frequent approach used to avoid this drawback and be safe to conclude that the effects are specific to the targeted gene.

DEFINING THE SIGNATURES OF CANCER CELLS: MICRORNAs PROFILING

Early detection together with the accurate description of the tumor type is crucial for a better diagnosis of cancer and a more effective therapy. Therefore, what is required to gain an increased survival rate of the patient is the identification of multiple biomarkers that can be measured simultaneously as a biological signature of the disease state. Using high-throughput technologies allows the identification of these signatures and their validation by the rapid comparison of samples from many different patients with the realistic hope of finding molecules that are informative of the type of cancer and with high predictive value for the patient.

The coordinated expression of specific miRNAs is believed to have a central role in diverse cellular processes, including cell proliferation and apoptosis, and their altered expression is involved in tumorigenesis. Indeed, expression profiling of differential miRNA has been shown to represent highly informative signatures for human cancers. Lu et al [25] developed a bead-based technique coupled to flow cytometry to determine the expression profiles of 217 human miRNAs in 332 cancer samples. They found that the expression pattern of a small set of miRNA dramatically varies across tumour types, reflecting the lineage and differentiation status of the tumor. In contrast, profiling expression data obtained on the same samples using 16,000 mRNAs was ineffective, thus implicating miRNA profiling as highly informative to classify human cancer types [25]. Furthermore, their results show that the expression pattern of a small set of miRNAs is highly informative to classify human cancer types.

Furthermore, as well illustrated by the three following recent papers, the development of microarrays containing all known miRNAs permitted to perform large-scale analysis of miRNA expression profiling in human cancers. In the first report the authors [26] evaluated the activity of miRNA genes in chronic lymphocytic leukemia (CLL) cells from 94 patients. Of the miRNA genes analysed, the researchers found that the activity pattern of 13 of them accurately predicted whether a person had the slow- or fast-progressing form of CLL. They also identified a germ-line mutation in the miR-16-1-miR-15 precursors, which caused low levels of miRNA expression and thus may be considered as cancer susceptibility genes for CLL (see below). In a second paper, by applying a similar approach, the same group examined miRNA expression profile in 76 breast tumors compared to normal breast tissue [27]. In this study 29 miRNAs were significantly deregulated in breast cancer (either over-expressed or downregulated). They found that miRNA expression was correlated with breast tumors' hormone status as well as its metastatic, invasive, and proliferative potential. Most important, their work demonstrated that the expression pattern of as few as five miRNAs (miR-10b, miR-125b, miR-145, miR-21, and miR-155) was sufficiently informative to successfully distinguish normal tissue from cancerous tissue. Finally, in the paper by Huiling He et al [28] the researchers examined samples of malignant tissue from 15 patients diagnosed with papillary thyroid carcinoma and compared them with the normal tissue adjacent to the tumors. They found 23 miRNAs that were significantly altered in the cancerous tissue when compared with the normal counterparts, with three of them (miR-146, miR-221, and miR-222) dramatically overexpressed, reaching 11-to-19-fold higher levels of expression in the tumors. Further investigation revealed that the expression pattern of miR-146, miR-221, and miR-222 if combined with that of two additional miRNAs (miR-21 and miR-181a), formed a "signature" that clearly predicted the presence of malignant tissue.

In conclusion, in several cancers the miRNA expression profiles seem to be sufficient to provide a "signature" that is directly associated to the clinical status of the disease. Indeed, the utility of single markers in diagnosis and monitoring of cancer is limited by the poor association of any single protein with a specific disease or stage of disease. Thus identifying the

distinctive signature of a network of these regulatory signals would enable us by a more precise diagnosis to detect tumors earlier, at times when treatments are more effective.

CANCER SIGNATURE MEASUREMENT

Developing methods that allow clinicians and researchers to translate signature discoveries to routine clinical use by looking simultaneously at a large number of biomarkers has now become a major challenge in cancer diagnosis. Indeed, because they are readily accessible without any need of invasive intervention measuring molecules expressed in serum or plasma is highly preferable. However, many potential cancer biomarkers in biological fluids are present at low concentrations, presumably in the low nanomolar range. Therefore, the capability to measure multiple protein markers simultaneously depends on methods having not only low limits of detection with elevated signals, but also coupled to very low noise, thus capable to distinguish specific protein signalling in the presence of a huge excess of unrelated proteins.

The use of aptamers as biorecognition element for the development of biosensors to detect protein targets offers over classical methods mainly based on antibodies, a lot of advantages, such as the possibility of easily regenerate the immobilised aptamers, their homogeneous preparation, and the possibility of using different detection methods due to easy labelling [29]. Moreover, the enormous diversity of random oligonucleotide libraries can exceed the diversity of antibodies in the mammalian genome by several orders of magnitude. Since aptamers are nucleic acids, experience with DNA, as in the production of DNA arrays, should be applicable to the development of aptamer-based biosensors. On the other side, the aptamer arrays can potentially expand the scope of DNA microarrays to recognise expressed proteins as well as expressed mRNAs. In this regard, numerous aptamers have already been selected against a wide array of proteins, and the possibility of acquiring aptamers against proteomes has been advanced by automation of the in vitro selection procedure. These considerations explain why now the aptamer-based technology for protein detection is in advanced stages of development as useful tools in clinical diagnosis and therapy. Furthermore, this technology has been improved by the use of modified aptamer molecules, named photoaptamers by Petach et al at SomaLogic, Inc [30–33]. These modified aptamers (either DNA or RNA) at specific locations include, in place of thymidine residues, the photoreactive 5-bromodeoxyuridine (BrdU) that can form a specific covalent crosslink with the target proteins. Indeed, short pulses of ultraviolet light at 308 nm induce a chemical crosslink between the BrdU residue and the electron rich amino acid on the target protein that is in a specific location in proximity and in the correct juxtaposition of the BrdU. Since this crosslinking event is dependent on the correct juxtaposition of the BrdU and the target amino acid, it conveys specificity to the photoaptamer-protein complex. This gives rise to multiplicative specificity by a photochemical cross-link that follows the initial affinity binding event. Proteins captured on the array are then measured either by universal protein staining or by using specific antibodies.

In order to measure simultaneously large numbers of proteins, even thousands, in biological fluids multiple capture photoaptamers can be deposited and covalently linked to the appropriate chip surface. Therefore, since photoaptamers covalently bind to their targets before staining, the photoaptamer arrays can be vigorously washed to remove background proteins, thus providing the needed potential for elevated signal-to-noise ratios.

The sensitivity and specificity of photoaptamers, combined with the ability to automate and scale up their selection and the ability to use them on solid surfaces, indicate that they could become an important factor in the development of proteomic technology.

IN VIVO DELIVERING

Whether being used as experimental tools and/or pharmaceutical drugs, small ncRNAs need to be able to cross cell membranes but negatively charged oligonucleotides will not pass through a lipid layer such as cell membranes. Similar to antisense oligonucleotides and ribozymes, the delivery of synthetic siRNAs and aptamers can be improved via the use of various delivery systems, which include synthetic carriers, composed primary of lipids. Larger carriers such as liposomes localise the drugs mainly to the blood compartment. However, angiogenic blood vessels in most tissues have gaps between adjacent endothelial cells large enough to cause the extravagation of liposome/siRNA complexes into the tumor. In addition, most solid tumors possess an enhanced vascular permeability and impaired lymphatic drainage, which leads to the accumulation of most liposomes within the tumor tissues. For these reasons, a big effort has recently been devoted to develop delivery vehicles that can efficiently deliver these RNAs molecules in vivo for the success of these molecules as therapeutics. Furthermore, the possibility to chemically modify and easily engineer small RNAs permits to express these molecules inside the target cell thus coupling the advantages of drug-based to those of gene-based therapy.

CONCLUDING REMARKS

Over the past few years the unexpected progress gathered in the knowledge of the mode of action of small noncoding RNAs is changing our point-of-view on the possible approaches to identify and to target tumor-associated genes. These flexible molecules have proven their enormous potential both as diagnostic and therapeutic tools in several fields of cancer medicine. Paradigmatic examples in the use of these small RNA molecules include that of siRNAs for high selective gene silencing, aptamers as high affinity inhibitory ligands, the miRNA profiling for a more accurate description of the tumor state, and the use of high sensitive aptamer-based biosensors to readily measure the proteomic status in biological fluids. Therefore, developing ncRNAs as the new generation of molecular tools for human health is an urgent

challenge that in the next future should provide us with the ability to tailor therapies to the cancer patient more effectively.

ACKNOWLEDGMENTS

This work was supported by the European Molecular Imaging Laboratory (EMIL) Network (LSHC-2004-503569), and by the MIUR-FIRB Grant (#RBIN04J4J7).

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