The International Journal of Biochemistry & Cell Biology 42 (2010) 1243–1251

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Review

RNA interference as a gene knockdown technique

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ARTICLE INFO

Article history:
Available online 12 May 2009

Keywords:
RNA interference (RNAi)
Systematic
Therapeutics
Morpholino
EGS

ABSTRACT

Not many scientific breakthroughs bring significant advances simultaneously in both basic research and translational applications like the discovery of RNA interference. Along with the elucidation of the RNA interference pathway and the discovery of its participation in crucial biological events, a branch of science has grown to utilize the RNA interference pathway as a biotechnology for both basic and applied research. Small interference RNA, plasmid-, and virus-encoded short-hairpin RNA are now regular reagents in the tool box of biologists to knockdown the expression of specific genes posttranscriptionally. Efforts have also been made to develop RNA interference based therapeutics into reality. Many concerns about the RNA interference technique have now been answered through research and development, although hurdles are still present. In this review, the RNA interference/microRNA pathway is briefly introduced followed with a detailed summary about the design and application of the RNA interference experiments, along with examples of the utilization of the RNA interference technology in animal cells and model organisms. Recent progresses and current concerns are also highlighted. Two techniques, namely morpholino and external guide sequence, are discussed as complementary gene knockdown technology. RNA interference technology, along with several other alternative gene knockdown techniques, is now indispensable to modern biological and medical research.

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1. Introduction

Fire, Mello and co-workers published their seminal work of RNA interference (RNAi) about a decade ago by revealing double-stranded RNA (dsRNA) as the trigger of post-transcriptional silencing in *Caenorhabditis elegans* (Fire et al., 1998). Another phenomenon of post-transcriptional silencing comes from microRNA (miRNA). In 1993, Ambros, Ruvkun and co-workers cloned the first short non-coding RNA (later called microRNA collectively), *lin-4*, also in *C. elegans*; and showed that *lin-4* potentially functions by binding to the 3′ UTR of its target, *lin-14*, through partial complementary sequences (Lee et al., 1993; Wightman et al., 1993). Advancement in the research eventually merged the RNAi pathway with the miRNA pathway by showing that core components are closely shared (Fig. 1; Murchison and Hannon, 2004).

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doi:10.1016/j.biocel.2009.04.023

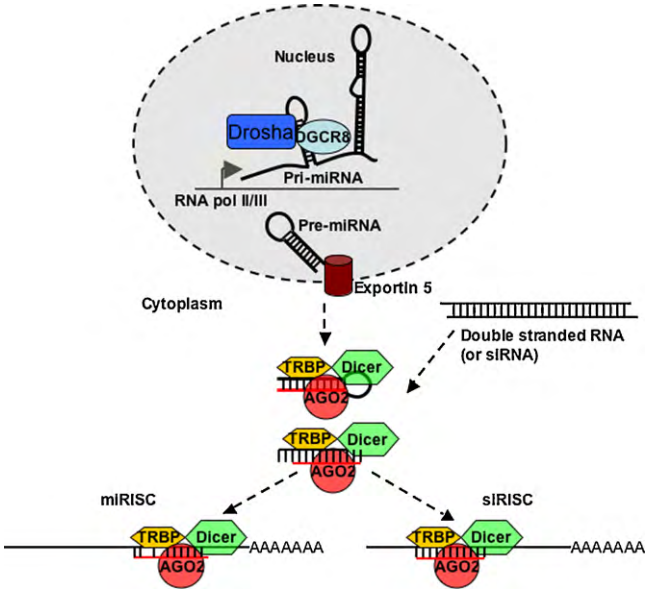

Fig. 1. The RNAi/miRNA pathway. miRNA genes are predominately transcribed by RNA polymerase II (some are transcribed by RNA polymerase III) into large primary miRNA (pri-miRNA) transcripts with poly(A) tail and 5′ cap. Pri-miRNAs are processed by a complex (called microprocessor) of DGCR8 and Drosha into miRNA precursors (pre-miRNAs) of ~70 nt with a hairpin structure of stem-loop. The formation of some pre-miRNAs bypasses the Drosh/DGCR8 because they are processed as intron (mirtron) in the pre-mRNA splicing (Ruby et al., 2007; Okamura et al., 2007). Pre-miRNAs are exported from nucleus into cytoplasm by Exportin 5, and get loaded into a complex composite of Dicer, Ago2, TRBP, and other known or unknown proteins. Double-stranded RNA (dsRNA) can also be loaded into this complex for processing. Pre-miRNAs or dsRNAs are cut into short miRNA duplex or small interference RNA (siRNA) around 21 bp by Dicer, and one strand (the guide strand, in red) is then be integrated into RNA-induced silencing complex (RISC) whose core component is Ago2. Another strand (the passenger strand, in black) will be degraded (for the siRNA duplex) or released (for the miRNA duplex). siRNA can also be loaded directly into the RISC complex without the need to slice by Dicer. Inside the siRISC (small interference RNA programmed RNA-induced silencing complex), the siRNA guide strand, by complete complementation with the targeted mRNA, triggers the degradation of mRNA in the RISC; whereas inside the miRISC (miRNA programmed RNA-induced silencing complex), miRNA generally suppresses the translation of the target mRNA by incomplete complementation with the 3′ UTR region. The whole pre-miRNA processing complex and RISC may be dynamic, share common components, and demand further characterization.

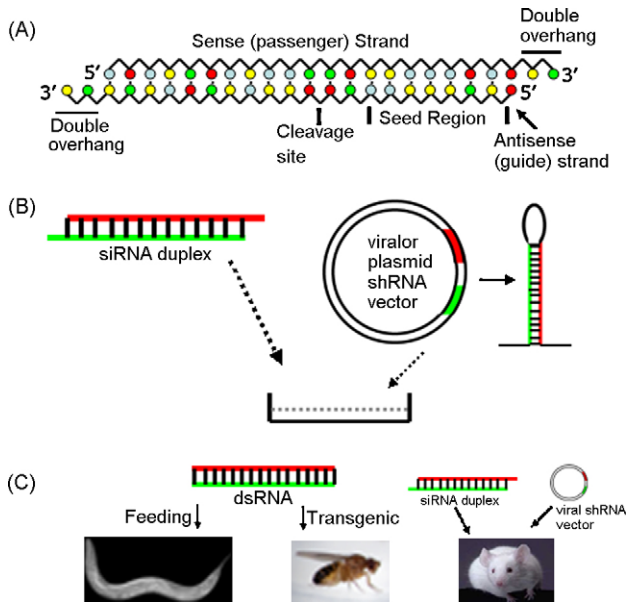

Fig. 2. Strategies of the RNAi technology. (A) Typical composition of a siRNA duplex with the passenger strand, the guide strand, seed region, and the cleavage site labeled. The first nucleotide (5′) of the guide strand is generally unpaired. siRNA duplexes can be chemically modified or conjugated for the purposes of increasing stability, avoiding immunostimulation, improving delivery, and enhancing potency. (B) siRNA duplexes or genetically encoded shRNA can be transfected (or transformed for viral shRNA) into mammalian cells with different methods. *Drosophila* S2 cells can also be transfected with dsRNA for RNAi knockdown. High throughput, whole-genome scale screening can be performed with cell cultures using siRNA, plasmid shRNA, or viral shRNA libraries. (C) RNAi can be applied to whole animals in *C. elegans*, *Drosophila*, and mice. Bacteria expressing dsRNA coded by a plasmid can be fed to *C. elegans* for RNAi knockdown; whereas dsRNA encoded by transgene can be induced to express in *Drosophila*. siRNA and viral shRNA can by applied to mice, and proper chemical modifications and delivery strategies should be considered. High throughput, whole-genome scale screening can also be done at whole animal level for *C. elegans* and *Drosophila*.

In principle, dsRNA, or microRNA precursor is cut into short interference RNA (siRNA) or miRNA duplex around 21 nucleotides (nt) by Dicer and its associated proteins such as TRBP. One strand (the guide strand, Figs. 1 and 2) is then be integrated into RISC. RISC is RNA-induced silencing complex whose core component is Ago2. Another strand (the passenger strand) will be degraded (for the siRNA duplex) or released (for the miRNA duplex). The siRNA guide strand, by complete complementation with the targeted mRNA, triggers the degradation of mRNA in the RISC; whereas miRNA generally suppresses the translation of the target mRNA by incomplete complementation with the 3′ UTR region (Fig. 1; Preall and Sontheimer, 2005).

Upon the discovery that dsRNA can be introduced exogenously into eukaryotic cells to knockdown target mRNAs in a sequence specific manner, a lot of laboratories started to design strategies to synthesis long dsRNA for later transfection experiments, or to construct plasmids encoding dsRNA for the knocking down of genes with special interest (Clemens et al., 2000; Tavernarakis et al., 2000; Montgomery et al., 1998). These attempts were largely successful in *C. elegans* and *Drosophila*, while inefficient in mammalian cells (Ui-Tei et al., 2000; Caplen et al., 2000). Reports also came out about long dsRNA triggering innate immune responses and cytotoxicities (Stark et al., 1998; Minks et al., 1979). Upon the discovery that RNA interference is mediated by ~21 nt small RNAs, and further the demonstration that siRNA duplex ~21 base pairs (bp) long can be applied to knock down gene expression in both *Drosophila* and mammalian cell cultures without triggering unwanted immune responses and cytotoxicity by Tuschl and co-workers, the RNAi technique became more practical (Zamore et al., 2000; Elbashir et al., 2001a,b). Today, the majority of RNAi knockdown experiments are performed directly with siRNAs or plasmid-/virus-encoded RNAs that eventually give rise to siRNA *in vivo*.

2. Design of siRNA

There are currently two ways to harness the endogenous RNAi pathway for gene knockdown purposes: either by introducing a viral or plasmid vector to express short hairpin (shRNA) that would then be processed by Dicer into siRNA (Abbas-Terki et al., 2002; Kunath et al., 2003); or by delivering directly siRNA into the cytoplasm (Elbashir et al., 2001a,b; Fig. 2). shRNA, which mimics a miRNA precursor, is usually expressed under a mammalian H1 or U6 promoter in a viral or plasmid vector. For either method, RNAi technique starts from the choosing and designing of one or several functional siRNAs. The first consideration is to ensure the siRNA targets specifically to the mRNA of interest without unwanted off-target effects (specificity), and at the same time, the siRNA should have a desirable (if not the highest possible) knockdown efficiency (potency).

The initial siRNA design starts with a bioinformatics-aided search for 'targetable' sequences of ~21 nt long in the mRNA of interest (Pei and Tuschl, 2006). Because a perfect complement with the targeted mRNA triggers degradation, and an imperfect comple-

ment triggers translational suppression, the possibility for a siRNA of ~21 nt to generate an off-target effect is high without careful design (Jackson et al., 2003; Lin et al., 2005). The principle here is to avoid complementary sequences in the siRNA 'seed region' to untoward mRNAs (Fig. 2). The 'seed region' is at positions 2–7 (or 2–8) of the guide strand of the miRNA or siRNA duplex. For miRNA function, the seed region usually perfectly complements the 3′ UTR of target mRNA (Lai, 2002). Off-targeting of siRNA is found to be associated with the presence of one or more perfect complementation of the seed region with the 3′ UTR of untoward mRNA (Birmingham et al., 2006). Several softwares and Internet search programs can be helpful in the selection of siRNA sequences for avoiding off-target effects (Qiu et al., 2005; Naito et al., 2005). These programs can be found from some websites such as http://dsCheck.RNAi.jp/ (to check and avoid sequences with high homology, Naito et al., 2005), http://rnai.cs.unm.edu/rnai/off-target/sirna_freq/, http://rnai.cs.unm.edu/rnai/off-target/genes-targeted/ (to check off-target effects, Qiu et al., 2005), and http://www.broad.mit.edu/genome_bio/trc/publicSearchForHairpinsForm.php (to search pre-evaluated siRNA sequences for mouse and human genes).

Although current understanding of RNAi activity cannot provide us with a precise prediction of potency to each individual siRNA, algorithms based on common features from high potency siRNA tested empirically are available (Pei and Tuschl, 2006; Reynolds et al., 2004). Once the bioinformatic part is done, candidate siRNA can then be synthesized and tested in cell culture systems for knockdown efficiency. The off-target effect can also be checked with a microarray assay. The final goal of this stage is to identify several siRNAs that show high knockdown efficiency and minimal off-target effect at nanomolar or lower concentrations.

Theoretically, there is an alternative way for selecting siRNA with high specificity and potency. Using a library of tiling siRNAs covering the whole mRNA sequence of interest can be screened with cell culture systems, and individual siRNA from this library with stringent specificity and high efficacy can then be identified. This method is more expensive and labor intensive, although knowledge gained from this approach can assist future design of siRNA.

When considering specificity and potency, it is beneficial to think about sequence conservation between species at the same time. siRNAs often have to go through tests with cell cultures from different species and animal models, so it is more reasonable to start with siRNAs that target conserved sequences in the mRNA.

Several practices can enhance specificity and potency of siRNA once its sequence is decided. For example, because the loading of siRNA into RISC is asymmetric (Schwarz et al., 2006; Khvorova et al., 2003), the first nucleotide of the 5′ end of the guide strand is usually made unpaired so that this strand can be loaded into RISC preferentially to increase its specificity and efficiency (Fig. 2).

siRNA can also be modified chemically to increase specificity and potency. Chemical modifications of riboses in the guide strand were found to suppress off-target effects without affecting the potency (Jackson et al., 2006; Fedorov et al., 2006). For example, 2′-O-methyl modification at nucleotide 2 of the guide strand is efficient to suppress off-targeting.

Modifying siRNA chemically can also increase its stability in the cells or animal/human body. Knowledge of avoiding nuclease degradation from previous research using antisense oligonucleotides and aptamers has been beneficial in the chemical modification of siRNA. Protection from nuclease degradation can be achieved mainly by two kinds of modifications: phosphodiester modifications and 2′-sugar modifications.

For example, replacing one of the two non-bridging oxygen atoms with a sulfur atom (P=S) or an isophosphonate borane ($-BH_3$) moiety is found to protect siRNA from exonuclease degradation (Layzer et al., 2004; Choung et al., 2006; Allerson et al., 2005; de Fougerolles et al., 2005). Moderate P=S modifications are also well tolerated in term of knockdown potency and toxicity. Also, modifications at the 2′ position of the ribose ring protect siRNA from endonuclease degradation. These mainly include 2′-O methyl (2′-OMe), 2′-deoxy-2′-fluoro (2′-F) modifications, and locked nucleic acid (LNA) (Chiu and Rana, 2003; Jackson et al., 2006; Allerson et al., 2005; Bondensgaard et al., 2000; Braasch and Corey, 2001). Appropriate 2′-OMe modifications not only increase the plasma stability but also alleviate off-target effects, and at the same time enhance *in vivo* potency of siRNA (Chiu and Rana, 2003; Jackson et al., 2006). Several recent reviews provide a more thorough summary on chemical modifications of siRNA (Watts et al., 2008; Corey, 2007).

Table 1
Examples of siRNA sequence and the corresponding knockdown efficiency.

siRNA sequence	Targeted gene	Knockdown efficiency	Notes and reference
GUUUUCACUCCAGCUAACAdTdT	CXCR4 (3′ UTR)	~80%	Sense strand (passenger strand) sequences were shown. Efficiencies of knockdown in cell culture at the mRNA level were shown. Alemán et al. (2007)
GCACGGAAGUCCAUCUGAAUU	Ago2	~93%	
GCAGGACAAAGAUGUAUUAUU	Ago2 (two mismatches)	~91%	
GUGCCUAUGUCUCAGCCUCUU	TNF-α	~60%[a]	Sense strand sequences were shown. Knockdown efficiencies were for protein level. Sørensen et al. (2003)
GAUCAUCUUCUCAAAAUUCUU	TNF-α	~40%[a]	
GACAACCAACUAGUGGUGCUU	TNF-α	~85%[a]	
GGAGAAAGUCAACCUCCUCUU	TNF-α	Not significant[a]	
GGCCUUCCUACCUUCAGACUU	TNF-α	~55%[a]	
GUGCCUAUGUCUCAGCCUCdTdT combined with GACAACCAACUAGUGGUGCdTdT	TNF-α	~80%[b]	
UGCCUCAGCGCCCCUUUGCdTdT	PKCα	~90%	Antisense strand (guide strand) sequences were shown. Knockdown efficiencies were for protein level. Leirdal and Sioud (2002)
AAACGUCAGCCAUGGUCCCdTdT	PKCα	~50%	
GCAGUGACCCACAGUGAUCUU	PKCα	~50%	
GGACUUGAAGAAGUCGUGCUU	GFP	~60%	
GAAGAAGAUGGUGCGCUCCUU	GFP	~70%	
GGCGGCUUUGCCAAGUGCUUdTdT	PLK	~30%	Sense strand sequences were shown. Knockdown efficiencies were for mRNA level. Reynolds et al. (2004)
AUGUCCAUGGAAAUAUCCAUdTdT	PLK	~45%	
GAUUGUGCCUAAGUCUCUGCdTdT	PLK	~70%	
UGAAGAUCUGGAGGUGAAAAdTdT	PLK	~50%	
AGGGGGGAGCCAAAAGGGUCdTdT	GAPD	Not significant	
UCAUCAUCUCUGCCCCCUCUdTdT	GAPD	~70%	
GAGCCAAAAGGGUCAUCAUCdTdT	GAPD	~90%	
CAAAAGGGUCAUCAUCUCUGdTdT	GAPD	~95%	

[a] Knockdown in cell cultures.
[b] Knockdown in mice.

Another concern in the design and synthesis of siRNA is avoiding innate immune responses, which nuclear acids can trigger (Uematsu and Akira, 2007). dsRNA longer than 30 base pairs can efficiently trigger serine/thronine protein kinase PKR. siRNA is smaller, but at higher concentrations, may trigger this pathway leading to a global blockade of translation and eventually cell death (Gitlin et al., 2002; Persengiev et al., 2004). Another issue, perhaps with greater concern in RNAi therapeutics, is that siRNA is potentially able to activate Toll-like receptors (TLRs), especially the dsRNA receptor TLR7 in plasmacytoid dendritic cells, that trigger the production of type I interferons and pro-inflammatory cytokines, and induce nuclear factor-κB (NF-κB) activation (Sledz et al., 2003). Some siRNAs have a higher tendency to activate TLR, and for this reason, they could be called isRNA (immunostimulatory RNA) (Hornung et al., 2005; Judge et al., 2005). Small RNAs with 3′ blunt ends and GU rich sequences are strong isRNA, which then should be avoided when designing the siRNA (Schlee et al., 2006). Chemical modifications at the 2′ sugar can also be beneficial because they help to avoid the immunostimulation (Sioud, 2008; Faria and Ulrich, 2008).

Research in designing siRNA with various improvements is still ongoing. There are reports on the benefits of longer siRNA duplexes, shorter siRNA duplexes, asymmetric passenger strands, etc. (Sun et al., 2008; Kubo et al., 2007; Chang et al., 2009).

To a lot of laboratories, the designing and synthesis of siRNAs or shRNA vectors are now relegated to companies such as Thermo Fisher Scientific® and Applied Biosystems®. Nevertheless, commercial pre-designed and validated siRNAs or shRNA constructs produced with the same principles discussed above are well accepted for general projects with cell culture systems with mouse, rat, or human origin.

RNAi technology is now being applied in many organisms in a variety of ways. This review concentrates on its application in animals, and readers can refer to other articles about the application of RNAi technology in plants (Fu et al., 2007; Baulcombe, 2004; Travella et al., 2006). To give an overall taste about the siRNA sequence and the corresponding knockdown efficiency, some examples of sequences, targeted genes, and knockdown efficiencies from several publications are summarized in Table 1.

3. Application of RNAi technique in cell cultures

The most common use of the siRNA technique is to knock down the expression of individual genes in cell cultures (Fig. 2). Generally the siRNA duplex is used for short term knockdown, and plasmid or viral shRNA vectors are applied for longer term knockdown, or for establishing stable cell lines. siRNA duplex and plasmid shRNA vector are usually transfected with commercial transfection reagents, which are actually lipoplexes. Viral shRNA vector is generally a good choice for hard-to-transfected or delicate cells that cannot stand chemical transfection. There are also efforts in using peptide-conjugated siRNA duplexes for high efficient delivery of siRNA into cultured cells without the need of lipoplex or liposome transfection reagents (Turner et al., 2007). Both 5′- and 3′-ends of the passenger strand are well tolerant to conjugations. The simplest peptide conjugation uses cholesteryl oligo-D-arginine (Chol-R9). Non-covalent forming of complexes of siRNA with Chol-R9 efficiently delivered siRNA targeting to VEGF into cells (Kim et al., 2006). Other peptides having been investigated include MPG, derived from the fusion peptide domain of HIV-1 gp41 protein and the nuclear localization sequence (NLS) of SV40 large T antigen. In cell culture, the peptide enables rapid delivery of the siRNA into the cytoplasm and results in robust down-regulation of target mRNA (Simeoni et al., 2003). Similar to MPG, penetratin, a peptide derived from the homeodomain of *Drosophila* protein Antennapedia, has been known to be able to deliver cargos into cells. Penetratin 1 conjugated siRNA can be delivered rapidly and efficiently into cultured primary mammalian hippocampal and sympathetic neurons (Davidson et al., 2004). TAT peptide (derived from the trans-activating transcriptional activator from HIV-1 virus) and other cell penetrating peptides (CPPs) have also been tested with various delivery efficiencies (Turner et al., 2007).

A particular concern in using the RNAi technique is the choice of negative and positive controls. Initially, untreated cells and mock transfection (or transformation for viruses) can be used as negative controls to examine the effects of the transfection process to cells and to the gene of interest. A well-accepted negative control for siRNA duplexes is an RNA duplex with a scrambled sequence, which has no perfect match in the genome. A negative control for plasmid or viral construct is usually the empty vector. Positive controls are applied to show that the RNAi experiment works in your particular cells with your RNAi protocol. A well pre-evaluated siRNA or vector can serve as a positive control. Issues about controls will be discussed further in Section 7.

The knockdown effect of RNAi can be checked with northern blot or real-time PCR at the mRNA level, and further with Western blot at the protein level. The RNAi protocol can be optimized based on evaluation of the knockdown efficiency and by referencing negative and positive controls.

4. Systematic application of RNAi technology

A growing interest is to exploit RNAi technology for systematic biology and functional genomics research to knockdown gene expression in whole-genome or whole-pathway scales.

4.1. C. elegans

dsRNA-mediated RNAi can be conveniently applied in *C. elegans* for knocking down gene expression of individual genes, or at a large whole-genome scale, to identify candidate genes for crucial biological events. In *C. elegans*, injection of dsRNA into the gonad of an adult hermaphrodite leads to a high frequency of the appropriate mutant phenotypes in her progeny, and in addition, *C. elegans* is so susceptible to dsRNA that merely soaking animals in the dsRNA or feeding them bacteria that produce dsRNA can lead to a mutant phenotype (Timmons and Fire, 1998; Tabara et al., 1998; Fig. 2). A whole-genome RNAi feeding library has been developed, and the feeding strategy is straightforward enough to be adapted for specific projects. It is now a prevalent method in the community of *C. elegans* (Kamath et al., 2001; Simmer et al., 2003). Many successful systematic RNAi screenings have been performed (Kim et al., 2005; Parry et al., 2007; Mabon et al., 2008). With the availability of several RNAi "super-sensitive" strains, RNAi knockdown in *C. elegans* can now be efficient for genes expressed in neurons, where exogenously induced RNAi is refractory for *wildtype* animals (Wang et al., 2005; Simmer et al., 2002; Kennedy et al., 2004; Samuelson et al., 2007). In *C. elegans*, RNAi can even be fine tuned for a specific developmental stage (Shan and Walthall, 2008).

The *C. elegans* community has enthusiastically employed this very successful feeding RNAi library, and so far, many whole-genome high-throughput screenings have been done to identify genes in crucial biological pathways such as fat regulatory, aging, and miRNA function (Ashrafi et al., 2003; Samuelson et al., 2007; Parry et al., 2007).

4.2. Drosophila

RNAi technology has now been used for several whole-genome screens in *Drosophila* using specific cell-based assays for a variety of pathways with dsRNA libraries (Kuttenkeuler and Boutros, 2004;

Boutros et al., 2004). DsRNA fragments are readily internalized into *Drosophila* S2 cells by scavenger receptor-mediated endocytosis (Ulvila et al., 2006), and most large scale RNAi screenings are performed with S2 cell cultures (Hao et al., 2008; Fleming and Rieder, 2003; Lents and Baldassare, 2006; Cherry, 2008). Genes related to cytokinesis, cell cycle regulation, specific signaling pathways, and virus replication are identified with S2 cells through RNAi screening (Björklund et al., 2006; DasGupta and Gonsalves, 2008; Mattila et al., 2008; Bonaldi et al., 2008; Hao et al., 2008).

Whole-genome RNAi can also now be performed in the whole organism of *Drosophila* with the availability of a genome-wide library of RNAi transgenes for conditional gene inactivation in *Drosophila* melanogaster (Dietzl et al., 2007; Fig. 2). This library allows the analysis and screening of genes playing roles in neurons and other cell types at the whole animal level (Liu and Davis, 2009; Cauchi et al., 2008).

4.3. Mammalian cells

siRNA libraries, plasmid-based shRNA libraries, and virus-based shRNA libraries are now available for high-throughput screening with mouse or human cell cultures (Fewell and Schmitt, 2006; Ganesan et al., 2008; Root et al., 2006; Chang et al., 2006). We have seen many successful examples of genome-wide RNAi-based functional genomics in identifying novel genes in pathways such as pigmentation, cell division, cell signaling, and virus replication (Ganesan et al., 2008; Kittler et al., 2007; Zhou et al., 2008). RNAi screenings have just been adapted to the whole organism level in mice in a successful *in vivo* screen with a shRNA pool to identify tumor suppressors in liver cancer (Zender et al., 2008).

For high-throughput screenings, organism and project specific considerations should be carefully addressed. Several recent publications provide further reference on this matter (König et al., 2007; Krausz, 2007; Lee, 2006; Paddison, 2008).

5. Application of RNAi to mammalian body

Direct application of the RNAi technique to the whole body or to a specific tissue/organ is being increasingly pursued for both basic and translational research. siRNAs or shRNA constructs verified with cell cultures can be delivered into animal bodies to investigate the effect of a specific gene in physiological context or to treat a specific disease with an animal model (Fig. 2).

For *in vivo* application, the central considerations are chemical modification and delivery strategy. Appropriate chemical modifications help siRNA duplexes to avoid immuno-stimulation and to withstand degradation from nucleases. Efficient and tissue specific delivery may be the biggest challenge in the application of RNAi to whole animals.

Different delivery strategies have been developed to meet the requirements for different disease model and target tissues (or organs). For viral shRNA vector, it is straightforward to apply the virus particles directly to the targeted tissue or organ. For siRNA duplexes, delivery has to be put under specific consideration. First, naked siRNA can be applied directly to organs such as the eye, lung, or central nervous system (CNS) through injection, instillation, or infusion (Bitko et al., 2005; Zhang et al., 2004; Dorn et al., 2004). Certain cell types can efficiently take up naked siRNA through unknown mechanisms, whereas many other cell types are refractory to naked siRNA. For this reason, siRNA duplexes have to be conjugated or formulated for efficient delivery in most cases. siRNA duplex can be delivered via liposomes or lipoplexes into the body. Liposomes are vesicles enclosed by a phospholipid bilayer, and can fuse with a cell membrane to deliver the enclosed contents into the cytoplasm. Both liposomes and lipoplexes have been successfully used to deliver siRNA into mice (Reich et al., 2003; Nakamura et al., 2004; Miyawaki-Shimizu et al., 2006; Luo et al., 2005).

siRNAs can be chemically conjugated with other molecules for *in vivo* delivery. The idea of covalently conjugating small molecules such as cholesterol was borrowed from previous research based on antisense oligonucleotide therapeutics (Eckstein, 2007). 5′-End cholesterol conjugation of the passenger strand has been shown to efficiently deliver siRNA intravenously for targeting the ApoB gene in the liver and jejunum (Kumar et al., 2006). RNA aptamers can also be conjugated to siRNA for the purpose of cell specific delivery. Aptamers are artificial DNA or RNA molecules that bind to specific molecular targets (Que-Gewirth and Sullenger, 2007). One report using a siRNA conjugated with a RNA aptamer that has a high affinity to prostate-specific membrane receptors showed that it silenced survival genes in prostate cancer cells with a high cell specificity and efficacy (McNamara et al., 2006). When it comes to cell specific delivery, peptides or antibodies can also be conjugated non-covalently with siRNA. A fusion protein with the specific antibody and a protamine fragment, which is arginine-rich and thus positively charged, can bind to siRNA (negatively charged) for delivery. Successful *in vitro* and *in vivo* applications include delivery of siRNA to B16 melanoma expressing HIV envelope protein or HIV-infected primary CD4 T cells using a fusion protein of protamine and Fab fragment of antibody to HIV envelope protein (Song et al., 2005). Peptide conjugations discussed previously in cell culture delivery section can also be used for *in vivo* delivery. One report showed successful *in vivo* delivery in animal model with chol-R9 (Kim et al., 2006). Some peptides can be recognized by cell specific receptors, and thus can be conjugated with siRNA for cell-specific delivery. Recently, a 29-amino-acid peptide derived from rabies virus glycoprotein (RVG) has been shown to be able to deliver conjugated siRNA (through a nonamer arginine fusion, RVG-R9) transvascularly to the brain resulting in efficient gene silencing. This peptide was found to bind specifically to the acetylcholine receptor expressed by neuronal cells (Kumar et al., 2007).

Another siRNA delivery strategy showing increased interest is the use of nanoparticles, which are structures in the size of nanometer scale (often 100 nm or smaller). Liposomal vesicles, lipoplexes, and antibody or some peptide-conjugated siRNA complexes discussed above are also actually nanoparticles. Nano-scale technologies are changing the foundations of drug delivery. These technological innovations are now referred to as nanomedicines by the National Institutes of Health. Nanoparticles have particular advantages in drug delivery. First, therapeutic agents can be encapsulated, thus, protected from degradation, clearance, and non-specific binding. Second, the release speed and location (tissue or even subcellular localization) of drugs can be well controlled by manipulation in the composition of nanoparticles. Third, the pharmacokinetics of the drugs can be optimized (Moghimi et al., 2005). At present, the most wildly used nanoparticle for siRNA delivery is a nanopolymer formed with polyethylenimine (PEI). A number of reports using PEI polymers demonstrated efficient siRNA delivery in animal models of influenza, Ebola virus infection, and tumors (Grzelinski et al., 2006; Urban-Klein et al., 2005; Ge et al., 2004; Tan et al., 2005). Components such as peptides and small compounds (e.g. folate) for cell specific delivery can also be added into the PEI-siRNA polyplexes. For example, arginine-glycine-aspartic acid (RGD)-peptide, which has been shown to bind to both tumor and tumor-endothelial cells *in vivo* (Zitzmann et al., 2002), has been successfully used to deliver PEI-siRNA into tumor tissues (Schiffelers et al., 2004). More information about the delivery of siRNA with nanoparticles is available in recent reviews (Juliano et al., 2008; de Fougerolles, 2008; Howard and Kjems, 2007).

Different delivery strategies can be used in combination. For example, siRNA can be conjugated covalently with small compounds and noncovalently with other molecules simultaneously,

and at the same time packed into liposomes or other nanoparticles with or without components (e.g. antibody and peptides) for tissue specific delivery.

6. Therapeutics

One of the ultimate goals of RNAi technology is to develop a new generation of drugs that target once thought "untargetable" mRNAs related to diseases. RNAi-based therapeutics begins with the design and identification of siRNAs that show high specificity and knockdown potency in research labs. Once several siRNAs are selected, they are then applied to animal models with diseases of interest. siRNA may need to be modified for its stability and then delivered via different routes and strategies discussed above. Highly potent siRNAs tested with animal models may then advance into clinic trials. Currently, several clinic trials are in place for treating diseases such as macular degeneration (AMD), diabetic retinopathy, and hepatitis C (de Fougerolles, 2008). More detailed discussions about RNAi-based therapeutics can be found from recent reviews (Shan and Jin, 2009; Haussecker, 2008).

7. Current concerns, hurdles and future promises

Several previous concerns around RNAi technology such as off-targeting and immunostimulation have been investigated very carefully. With more knowledge about the "seed region" of miRNA and the properties of isRNA, these concerns are now well addressed, and off-targeting and immunostimulation can be managed to minimal or acceptable levels through optimal siRNA designing and modifications. RNAi technology utilize the endogenous RNAi/miRNA pathway, and one concern still persisting is that exogenous siRNA may exaggerate components in the RNAi/miRNA pathway from their endogenous functions. Indeed, Grimm et al. reported using adeno-associated virus type 8 as a viral vector for shRNA expression in liver (Grimm et al., 2006). Lethality was found to be widespread at higher viral titers due to over-saturating the endogenous miRNA pathway. Thus, the application of adenovirus for RNAi to whole animal (and for any RNAi experiment in general) has to be very careful in optimizing viral dosage and sequence of the encoded shRNA. This concern of exaggerating endogenous RNAi/miRNA functions also need to be addressed when setting up negative and positive controls. Although scrambled RNA (for siRNAs) or empty vector (for shRNA constructs) are acceptable negative controls, one should keep in mind that these controls would not utilize the RNAi pathway at all, and thus do not take up the RNAi pathway from its endogenous functions. I propose here that a functional irrelevant siRNA should be used at the same dosage as a negative control for specific projects. For example, a siRNA (or shRNA) for GAPDH can be used as a negative control in a project to knockdown P53 with RNAi. On the other hand, a functional relevant siRNA (e.g. targeting to a gene in the same pathway) should be used at the same dosage as a positive control. Dosage and effective periods still need to be improved for siRNA. Currently, nano- to micro-molar concentrations are applied to cell cultures and whole organisms. This is a well-accepted concentration range for most small molecular drugs. But for siRNA, it is still relatively expensive. Efforts have been undertaken to lower the required dosage and to increase the effective period for siRNA (Shan et al., 2008). Our knowledge in the function of the RNAi/miRNA pathway and other small RNA pathways has been increasing dramatically, while we are still far from recognizing the full effects of small noncoding RNAs. Caution and alternative verifications need to be applied when we explain the effects for any siRNA or shRNA, as a foreign introduced small RNA.

RNAi technology is used predominantly for targeting mRNA. More recently, some researchers applied RNAi to knockdown noncoding RNAs transcribed by pol II such as HOTAIR (Rinn et al., 2007). Whether RNAi works for noncoding RNAs generated by pol III and other ncRNAs is still an open question. With the dramatic expansion of the list of noncoding RNAs, RNAi and other technologies need to be evaluated for effectiveness in knocking down noncoding RNAs.

As a phenomenon biologists have started to uncover from the end of the last century, RNAi has generated enormous success for scientists in both basic and medical research. It enables a lot of projects that were unable to be pursued previously. The advancement of RNAi technology is rapid and has achieved breakthroughs with enormous enthusiasm and effort. In the near future, we will see more predominant usage of RNAi technology specifically in high throughput whole-genome screening and translational research to develop RNAi-based therapeutics. Combinatory use of systematic RNAi and proteomics or other high-throughput technologies is another trend currently under development (Selbach and Mann, 2006; Bauer and Ueffing, 2006; Kittler et al., 2008). With the emergence of more understanding of the RNAi/miRNA pathway itself and great advances in technique and strategy development, a great optimism exists in the success and miracles that RNAi technology will bring to biology and medicine.

8. Other methods for gene knockdown

Even though RNAi is now the dominant technology used in animals, it is better to keep in mind the existence and sometimes advantages of other alternative technologies for gene knockdown.

One gene knockdown technique based on antisense strategy called "morpholinos" has been applied for decades to mice, zebrafish, frogs, and sea urchins (Heasman, 2002). A morpholino oligo is a modified nuclear acid polymer (~25 bases) in which the ribo sugars are replaced with morpholine rings. Morpholinos are sometimes referred to as PMOs (phosphorodiamidate morpholino oligos). Morpholinos bind to the target mRNA through Watson/Crick base-pairing and block the translation (binding to the start codon) or splicing (binding to the exon/intron junction). Recently, morpholinos have been used successfully to block the maturation of specific miRNA (Flynt et al., 2007). Generally, morpholinos are very stable in cells and the blood stream, and considered to have minimal off-target and side effects (Summerton, 2007). Morpholinos dominate gene knockdown applications in developing embryos, specifically, in zebrafish. One genuine restriction in the application of morpholinos is that morpholinos cannot be encoded genetically, and get eventually diluted to below-effective concentrations during cell proliferations.

External guide sequence (EGS) is another technology developed for posttranscriptional knockdown of gene expression (Li et al., 1992; Yuan and Altman, 1994). It utilizes the endogenous RNase P enzyme to cleave target RNA under the guidance of an exogenously introduced RNA molecule (EGS RNA) (Li et al., 1992; Yuan and Altman, 1994; Ma et al., 2000). RNase P is an enzyme responsible for the maturation of tRNA by cleaving the 5′ of the tRNA precursors, and is universally present in cells from bacteria to mammals (Altman, 2007). One of the advantages of EGS technology derives from the fact that RNase P is universal; thus it can be utilized in almost all cells. EGS technology can essentially target any RNA species as long as the RNA of interest and the EGS RNA form a structure that mimics a tRNA precursor; therefore, can be used to target both mRNA and noncoding RNAs transcribed by all the RNA polymerases, which is clearly an advantage to exploit in the future. RNase P is relatively abundant in most cells and localized in the nucleus in eukaryotic cells, which ensures an early, efficient knockdown by EGS without occupying too much RNase P from

its endogenous functions. A plasmid encoded EGS targeting RPP38 mRNA in Hela cells can achieve comparable (if not higher) knockdown efficiency in much shorter periods (18 h versus 48 h) when compared with RNAi (Ge Shan, unpublished data).

Currently EGS technology has been applied with different degrees of success in a variety of prokaryotes and eukaryotes (Ma et al., 2000; Bassett et al., 2008; Ko et al., 2008; Lundblad et al., 2008). These include trials to inhibit pathogenic bacteria by down-regulating virulent genes, and to control virus infection *in vivo* in mice by degrading essential viral mRNA (Bai et al., 2008; Reyes-Darias et al., 2008). Although more research such as developing it into a streamline process for more convenient application, and evaluating thoroughly the off-target and side effects, has to be done before we can fully enjoy the advantages the EGS technology has to offer.

9. Conclusions

RNAi technology is a very timely invention in the era of postgenome to serve as one of the most powerful tools for reverse genetics, functional genomics, and systematic biology. Although we should keep in mind that continuous improvements and modifications are necessary to make RNAi technology more potent, it already revolutionizes our way of doing biology and medicine. Two lessons are learned here. First, old (sometimes less efficient) technology can aid the development of new technology. The explosion of RNAi technology actually benefited from the previous development of antisense technology. Second, basic research is essential to applied research. The discovery of the RNAi/miRNA pathway opens the door to RNAi technology, and further characterization of this pathway really facilitates the development of RNAi technology step by step.

Acknowledgements

The author would like to thank Dr. Sidney Altman and Donna Wesolowski for critical comments on the draft manuscript. Ge Shan is supported by grants to Dr. Sidney Altman.

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