EXPERT REVIEW

Subcellular Fate and Off-Target Effects of siRNA, shRNA, and miRNA

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ABSTRACT RNA interference (RNAi) strategies include double-stranded RNA (dsRNA), small interfering RNA (siRNA), short hairpin RNA (shRNA), and microRNA (miRNA). As this is a highly specific technique, efforts have been made to utilize RNAi towards potential knock down of disease-causing genes in a targeted fashion. RNAi has the potential to selectively inhibit gene expression by degrading or blocking the translation of the target mRNA. However, delivering these RNAs to specific cells presents a significant challenge. Some of these challenges result from the necessity of traversing the circulatory system while avoiding kidney filtration, degradation by endonucleases, aggregation with serum proteins, and uptake by phagocytes. Further, non-specific delivery may result in side-effects, including the activation of immune response. We discuss the challenges in the systemic delivery to target cells, cellular uptake, endosomal release and intracellular transport of RNAi drugs and recent progress in overcoming these barriers. We also discuss approaches that increase the specificity and metabolic stability and reduce the off-target effects of RNAi strategy.

KEY WORDS cellular uptake · intracellular trafficking · miRNA · RNA interference · shRNA · siRNA

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INTRODUCTION

In 1998, Andrew Fire and Craig C. Mellow demonstrated in a *C. elegans* model that double-stranded RNA (dsRNA) was more effective in silencing gene expression than the sense or the antisense strands (1). Later, this phenomenon was experimentally demonstrated in other species such as flies (2,3) and vertebrates (4–7). RNA interference (RNAi) has found widespread application in scientific research and holds the potential for revolutionizing medicine. Successful RNAi therapy is dependent upon various factor, the most critical being the ability to be delivered to the specific tissue in question. RNAi is an ancient mechanism that allowed eukaryotes to regulate endogenous gene expression and inhibit expression of viral genes using small interfering RNA (siRNA) (8–10). Strategies like antisense oligonucleotides (ODNs) and triplex forming oligonucleotides (TFOs) are much older than RNAi but are yet to gain the interest achieved by the latter as potential therapeutic agents. While 21–23 nucleotides long siRNA molecules can regulate multiple mRNA transcripts, antisense ODNs are limited to blocking the translation of only one copy of mRNA before being degraded (11,12). TFOs are antigene molecules that can bind to double stranded DNA and give rise to triple helical structures, thereby inhibiting initiation of gene transcription (13–16). A comparative *in vitro* study by Miyagishi and colleagues (2003) concluded that siRNA therapy was about 100-times more effective than an antisense directed against the same gene target resulting in siRNAs silencing target gene for longer duration (17). RNAi is mediated through a number of molecules including dsRNA, siRNA (19–23 base pairs), short hairpin RNA (shRNA), and microRNA (miRNA) (Fig. 1). While
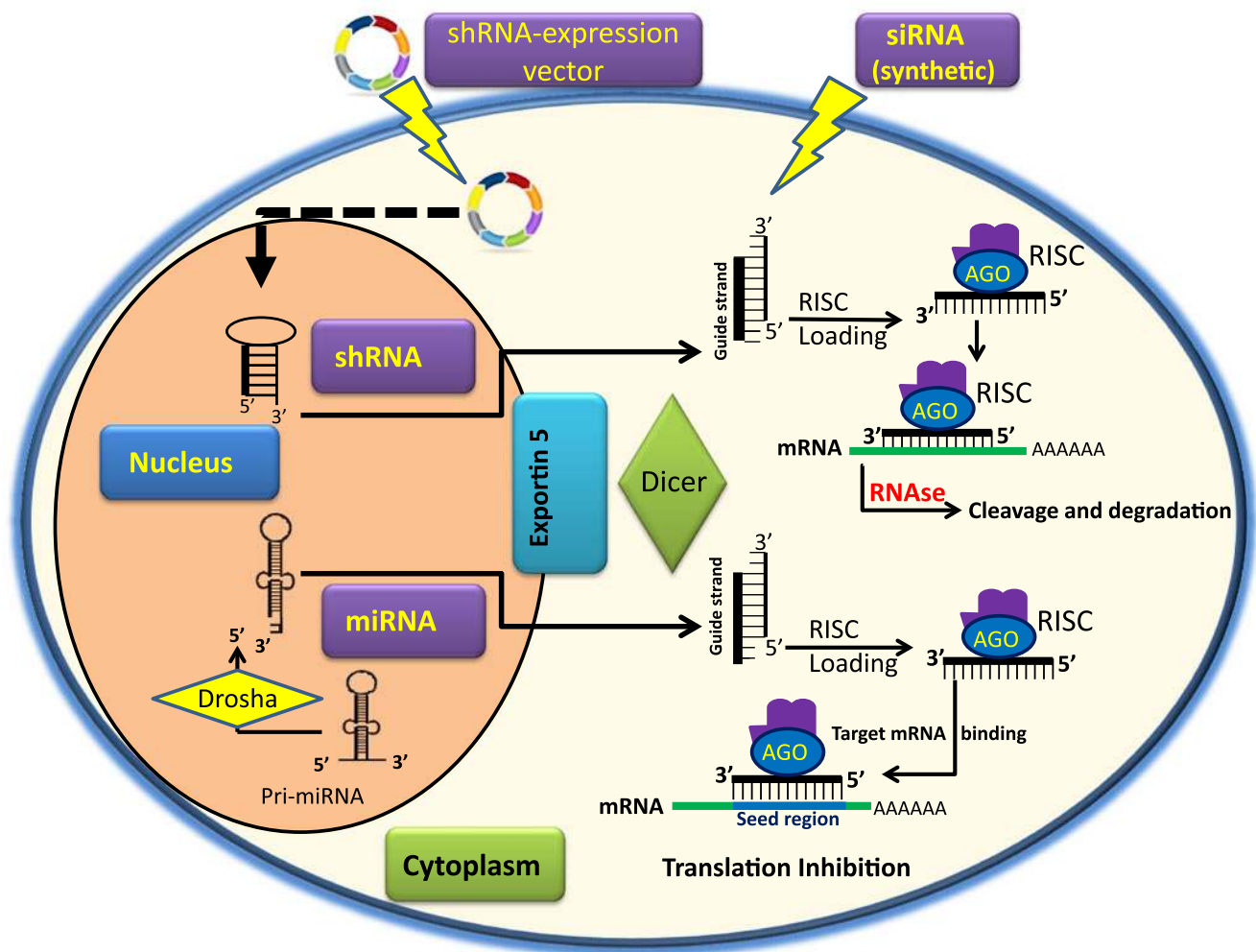
Fig. 1 Mechanism of RNAi. Double stranded RNA (dsRNA) is processed by Dicer into duplexes of 21 to 24 nucleotides long short interfering RNA (siRNA). siRNAs are incorporated into the multiprotein RNA-induced silencing complex (RISC), while synthetic siRNA is directly incorporated into the RISC without going through Dicer processing. RISC unwinds the duplex siRNA and one strand pairs to messenger RNAs (mRNAs) which has a high degree of sequence complementarity with siRNA. Argonaute-2 (Ago-2) in human RISC degrades the mRNA at sites not bound by the siRNA. Vector carrying shRNA enters the cell, crosses the cytosol gel and travels to the nucleus where it is transcribed into a hairpin like structure which is exported to the cytoplasm. shRNA, after moving to cytoplasm is processed similar to siRNA. The precursor of primary miRNAs (pri-miRNAs) is transcribed in the nucleus and is digested by dsRNA-specific nuclease Drosha to form another precursor miRNA (pre-miRNA) before being exported to the cytoplasm. Exportin-5 (Exp5) is the key protein responsible for the active movement of pre-miRNAs and shRNA from the nucleus to the cytoplasm. Like siRNAs, miRNAs are processed by Dicer creating the mature miRNA, one strand of which is incorporated into a RISC. Imperfect base pairing (mismatch) between the miRNA and its target causes RISC to either degrade the mRNA transcript or prevent its translation into protein.

being structurally different, all of these molecules have the capability to induce sequence-specific gene silencing.

First experimental treatments using RNAi based approaches were underway within three years of the discovery of this phenomenon (7,18,19). However, as dsRNA can elicit immune response such as activating dicer-related antiviral pathways, gene silencing in mammalian cells usually involves the use of siRNA (20). While siRNA is considered a promising molecule to treat various infectious, genetic and pathological diseases, as well as various types of cancers, there is no US FDA approved RNAi drug currently in the market. Much of this gap between potential and actuation results from various

barriers these therapies have to overcome before they can efficiently silence harmful/aberrant gene expression. These obstacles may be systemic, local or cellular and result in rapid excretion, low serum stability, nonspecific tissue accumulation, poor uptake by the cells and endosomal release into cytoplasm. In addition, for shRNA, the intracellular trafficking is another noteworthy barrier as the plasmid has to traverse through the gel-like cytoplasm in order to translocate to the nucleus [reviewed in (21–23)]. The efficacy of RNAi approach is further compromised by resulting side effects, including non-specific gene silencing, cytotoxicity, activation of immune response, RNA degradation by endonucleases, induction of apoptosis and the

inhibition of endogenous miRNA (22,24–27). Taken together, novel delivery systems that can reduce off-target effects, improve cellular uptake, and release siRNA drugs hold the key for successful gene silencing.

GENE SILENCING MECHANISMS

si/shRNA-Based Gene Silencing

RNAi can be mediated either through the introduction of long (500–1000 nucleotide) dsRNA strands, chemically synthesized exogenous siRNA or vector transported but endogenously processed and expressed RNA molecule Following their cleavage by an RNAse III enzyme (Dicer), the siRNA is created as 21-or 22-nt dsRNA fragments bearing 3′-overhangs of 2 nts on either strand (28). Dicer, which contains a catalytic domain in addition to helicase and PAZ domains (9,29,30), is required for both RISC formation as well as the entry of siRNA into RISC (31). These dsRNAs (now siRNAs), are assembled selectively into a RISC which has argonaute 2 (Ago2) as a functional unit for cleaving the passenger strand and releasing the guide strand from the duplex (32,33). The unwinding of the ds siRNA sequence yields a single strand 'guide' RNA that can entice RISC to its homologous (and therefore target) mRNA (34,35). After binding to this homologous mRNA, RISC cleaves it in the middle of the region bearing complementary base pairing to the guide strand. The guide is not cleaved and RISC can thus attach to and cleave multiple copies of target mRNA, leading to effective RNAi (29,36).

Even though their functional outcomes may be similar, siRNA and shRNA differ significantly in their processing, mechanisms of action and ultimately the RNAi pathways mediated by them (Fig. 1). shRNA has a hairpin structure that is generated by the processing molecules in the target cells (37). These shRNAs can be produced from plasmid or viral vectors designed for either transient or long-term gene silencing (38). Essentially, exogenous vectors encoding a shRNA construct is transfected into the cell, where it translocates to the nucleus where shRNA is expressed as pri-shRNA. Drosha, a nuclease converts pri- into pre-shRNA. A specialized nuclear membrane protein called exportin-5 (Exp5) transfers pre-shRNA to the cytoplasm, where Dicer processes it to functional siRNA capable of incorporating into a RISC to cleave the target mRNA (33,39).

In humans, the Ago2 protein associates with Dicer-HIV-transactivating response RNA-binding protein (TRBP) to form RISC-loading complex (RLC). Wang and colleagues have used functional human RISC-loading complexes (RLCs) to examine both the RLC and its Dicer units (40).

Their results strongly suggest that TRBP stabilizes RLC and prepares the siRNA for loading onto Ago-2 and thence the RISC. Finally, TRBP ensures the proper orientation of the siRNA following its loading, thereby implicating it as a major player in the selection and incorporation of the guide strand in RISC. As pre-shRNA has been shown to be associated with RLC, it is possible it does not get processed via a Dicer/TRBP complex and instead is directly attached to RLC. After loading onto RLC has occurred and the passenger strand of pre-shRNA is lost, si and shRNA RISC behave in a similar fashion.

miRNA-Based Gene Silencing

miRNAs are endogenous, noncoding RNAi molecules about 22 nt long and are capable of negatively modulating post-transcriptional expression of genes by binding to their complementary sequence in the 3' untranslated (UTR) region of mRNA targets (41). It is believed that almost 1000 miRNAs regulate expression of almost a third of the vertebrate genome (42). The first miRNA was identified in *C. elegans* by Lee and colleagues (1993) and was shown to downregulate a post-embryonic developmental protein (43).

miRNA, like siRNA, need to be incorporated into a RISC to cause RNAi. Unlike siRNA, miRNAs are generated in the nucleus and transported to the cytoplasm as mature, hairpin structures. The precursor units of miRNA in the nucleus are the pri-miRNAs, which are several kilobases in length and are transcribed by RNA polymerase II (44). These transcripts are processed by the RNAse III nuclease, Drosha, in tandem with double-stranded binding domain protein DGCR8 (known as Pasha in invertebrates) into 70 nt long loop-stem precursors (44,45). Processed pre-miRNAs are actively shunted to the cytoplasm by the GTP-powered transporter exportin 5 (Exp5) for second round of processing by the Dicer-TRBP microprocessor complex (28,46,47). This leads to the release of a dsRNA duplex which has the mature miRNA still attached to its complementary strand loaded into a RISC. The mRNA transcripts in miRNA induced RNAi are either degraded or have their translation blocked. This inhibition of translation involves a phenomenon termed the partial base pairing (or imperfect complementarity). For this to occur, the 'seed region' in the 5' end of the miRNA (a header of nucleotides 2–7) has to be perfectly complementary to the target mRNA (27,41,48).

miRNAs are involved in regulating a number of life processes including embryonic development, cell differentiation and proliferation, cell death and organismal response to stress (49). miRNA expression profiling has been used to demonstrate characteristic miRNA signatures in tumor tissue (50–52), suggesting that miRNAs may become useful

diagnostic tools (53,54). Endogenous miRNA can be inhibited through the introduction of antisense molecules capable of targeting either mature miRNA or by the introduction of siRNA/shRNA for blocking the miRNA processing cellular machinery. For example, Esau and colleagues inhibited miR-122 by systemically dosing normal mice with anti-miRNA antisense oligodeoxyribonucleotide (AMO), resulting in an increase in mRNA transcripts of miR-122 target genes (55). Even though the precise mechanism involved in the AMO-mediated miRNA down regulation is not known, AMO probably acts on mature miRNAs as no reduction in the levels of pre-miR-122 was reported. Recently, cholesterol-linked, single stranded RNA molecules called antagomirs, have been utilized to study the gene regulatory effects of miRNAs. These are 21–23 nts long and bear complementarity with the mature miRNA sequence. Successful use of cholesterol-linked antagomirs has enabled research into *in vivo* functions of miRNA in rodent models (56–58).

BARRIERS TO SYSTEMIC DELIVERY TO TARGET CELLS

Even though understanding the mechanism of RNAi therapeutics has made great advances, the systemic delivery of RNAi molecules remains a challenge (78). Local delivery offers the advantage of low effective doses and simple formulation that may not require targeting ligands or extensive chemical modifications (23). Also, the risks of systemic side effects are limited. For this reason, local delivery is currently an efficient strategy for *in vivo* siRNA mediated RNAi compared to systemic delivery. Most of the successful preclinical and clinical trials of RNAi drugs have been for local delivery into the affected tissue or organ. For example, clinical trials for treating age-related macular degeneration using RNAi molecules rely on the direct injection of siRNA into the eye (59). Other routes for local delivery include intranasal siRNA administration for pulmonary delivery (60,61) and injections into the brain (62).

Metastatic tumors are especially amenable for systemic delivery compared with local administration. Studies have shown that siRNA formulated in nanocarriers can be systemically delivered into rodent models, resulting in efficient and persistent antiviral and anticancer activity (63,64). The knockdown of oncogene Bcl-2 by systemically administered siRNA prevented tumor growth in a murine liver cancer model (65,66). Compared to local delivery, systemic siRNA delivery needs to cross several additional barriers. To be effective, siRNA should remain in its active form during circulation; avoid kidney filtration, phagocytic uptake, serum protein aggregate formation, and degradation by nucleases. Further, it needs to cross the vascular

endothelial barrier to reach tissues. While this barrier prevents molecules > 5 nm from making their way through, organs such as liver and spleen are amenable to the entry of molecules up to 200 nm diameter. In addition, tumors possess a 'leaky' and discontinuous vasculature which allows the penetration of high molecular mass macromolecules (>40 kDa), a phenomenon known as 'enhanced permeation and retention effect' (EPR) (67,68). Once the RNAi molecule has exited the bloodstream, it needs to traverse the extracellular matrix (ECM), a dense network of structural proteins and polysaccharides surrounding the target cell. ECM can significantly retard the uptake of the drug carrier, thus enhancing its phagocytosis and destruction (69).

SUBCELLULAR FATE OF RNAi MOLECULES

In addition to the need of efficient delivery to target cells, obstacles related to endosomal release, cytoplasmic stability and intracellular trafficking of RNAi molecules are the primary reasons for RNAi not living up to its potential. Figure 2 illustrates various biological barriers which RNAi molecules must overcome to confer efficient gene silencing.

Cellular Uptake and Endosomal Release

The plasma membrane is a major barrier for the uptake of si/sh-RNA. Their hydrophilic nature and high molecular weight as well as the net negative charge carried by siRNA molecules results in poor uptake efficiency. Several approaches can be utilized to increase their cellular uptake. For example, complexation of siRNA molecules with cationic lipids and polymers results in their partial condensation masks their net negative charge and imparts an overall positive charge to the complex leading to increased cellular uptake. Studies using fluorescent dye labeling of gene delivery vehicles and colocalization analysis with cellular compartments have suggested that the drug complex undergoes receptor mediated endocytosis and is deposited into early endosomes, which is the first step in the intracellular trafficking of siRNAs (70,71). Further, the exact endocytic pathway is dependent on the cell type and the gene carrier being utilized (70,72). The early endosomes combine with sorting endosomes, which fuse and with the more acidic (pH 5–6) late endosomes. The endosomal contents relocate to the lysosomes, which have even lower pH (approximately 4.5) and are the sites for nuclease-mediated siRNA degradation. To avoid lysosomal degradation, siRNAs (free or complexed with the carrier) must escape from the endosome into the cytosol (47,73). Endosomal escape is a major rate-limiting step in RNAi process.
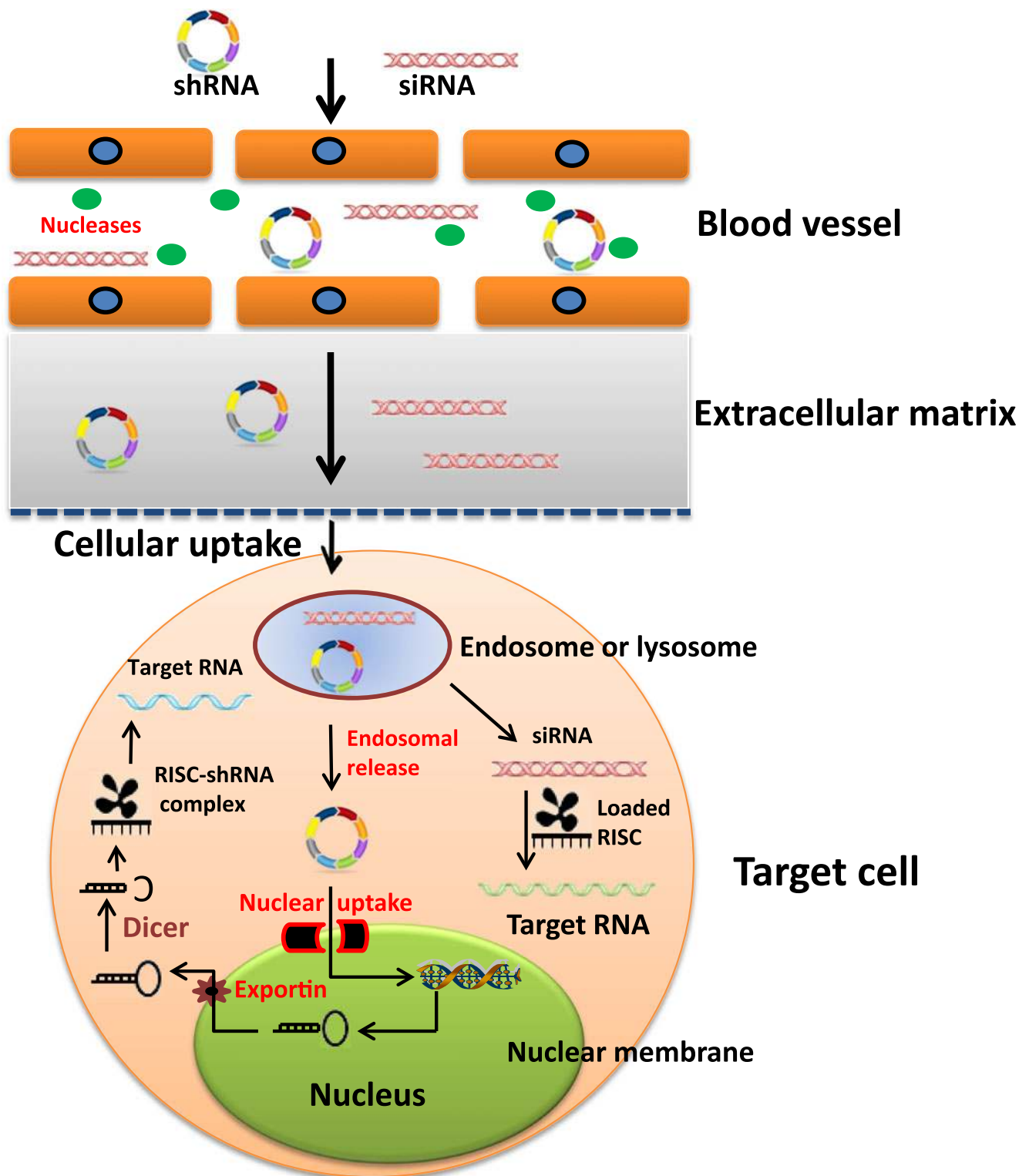
Fig. 2 Barriers for siRNA delivery. Following systemic administration, siRNAs come in contact with blood nucleases; then they traverse the blood vessel barrier and the extra-cellular matrix compartment prior to crossing the plasma membrane barrier. Upon entering the cell via receptor mediated endocytosis, they are trapped in endosomes and need to be released in the cytosol. Endosomal escape is a major rate limiting step in RNAi.

Strategies that promote endosomal release include the use of membrane-interacting peptides, fusogenic lipids and polymers with high buffering capacity. An *in vivo* targeting strategy has been described by Rozema and colleagues in liver cells. Their multifunctional polymer carrying a targeting ligand allows recognition by target cells. They

used N-acetylgalactosamine carrying poly(butyl vinyl ether), abbreviated as pBAVE, for *in vivo* siRNA delivery to hepatocytes. N-acetylgalactosamine which targets siRNA for uptake by hepatocytes is degraded by the low pH of the endosome. pBAVE is endosmolytic and carries the siRNA with it while escaping into the cytosol. Here, due to the reductive environment, the disulfide bonds joining siRNA to pBAVE are broken. The free siRNA can now interact with RISC and cause cleavage of its target mRNA (74).

There is a strong correlation between siRNA localization inside the cell and concomitant RNAi activity. Chiu and colleagues (2002) have demonstrated this correlation in HeLa cells which were transfected with fluorescent-tagged siRNA against CDK-9 gene.(75) As shown in Fig. 3, while siRNAs transfected using lipofectamine were primarily localized to perinuclear regions, those delivered using high concentrations of nanoparticles were found distributed to the nucleus and nucleolus. With this change in subcellular localization of siRNA, a decrease in the RNAi activity was observed, suggesting that RNAi is dependent on the subcellular fate of siRNA.

To further understand the trafficking and fate of siRNA delivered to the cell, Jarve and colleagues (2007) dual labeled a siRNA with a fluorescent dye pair and visualized it using fluorescence resonance energy transfer (FRET). Within 15 min of microinjection, the intact siRNA was observed in the nucleus while at 4 h, fluorescence was detected only in cytoplasm, suggesting complete translocation (76). The accumulation patterns of siRNA seemingly mirrored the behavior of antisense ODNs in the authors' previous studies where they had concluded that there was little or no active inbound transport through the nuclear pore (77). The dynamics of siRNA in cytoplasm of living cells were also explored by Shin and colleagues who demonstrated that after its introduction into the cells, the siRNA duplex gradually diffuses into cytosol, and is then degraded within 3.5 h (78).

Using siRNA targeted against viral mRNA, Berezhna and colleagues have demonstrated that RNAi machinery controls the localization of the siRNA to subcellular compartments, including the nucleus, if the target RNA is located there. (79). Studies such as this open the possibility that RNAi may involve both cytoplasmic and nuclear mechanisms. Ohrt and colleagues have identified two distinct RISC, of which the larger exists in the cytoplasm while a 20-fold smaller complex is found in the nucleus. They also demonstrated that nuclear RISC, which comprises only Ago2 and a small RNA strand, originates in the cytoplasm from cytoplasmic RISC and moves into the nucleus only if its target is located there. These results establish a novel mechanism for dynamic RISC movement between nucleus and cytoplasm for gene silencing in both organelles. Since siRNA and miRNA lack a nuclear
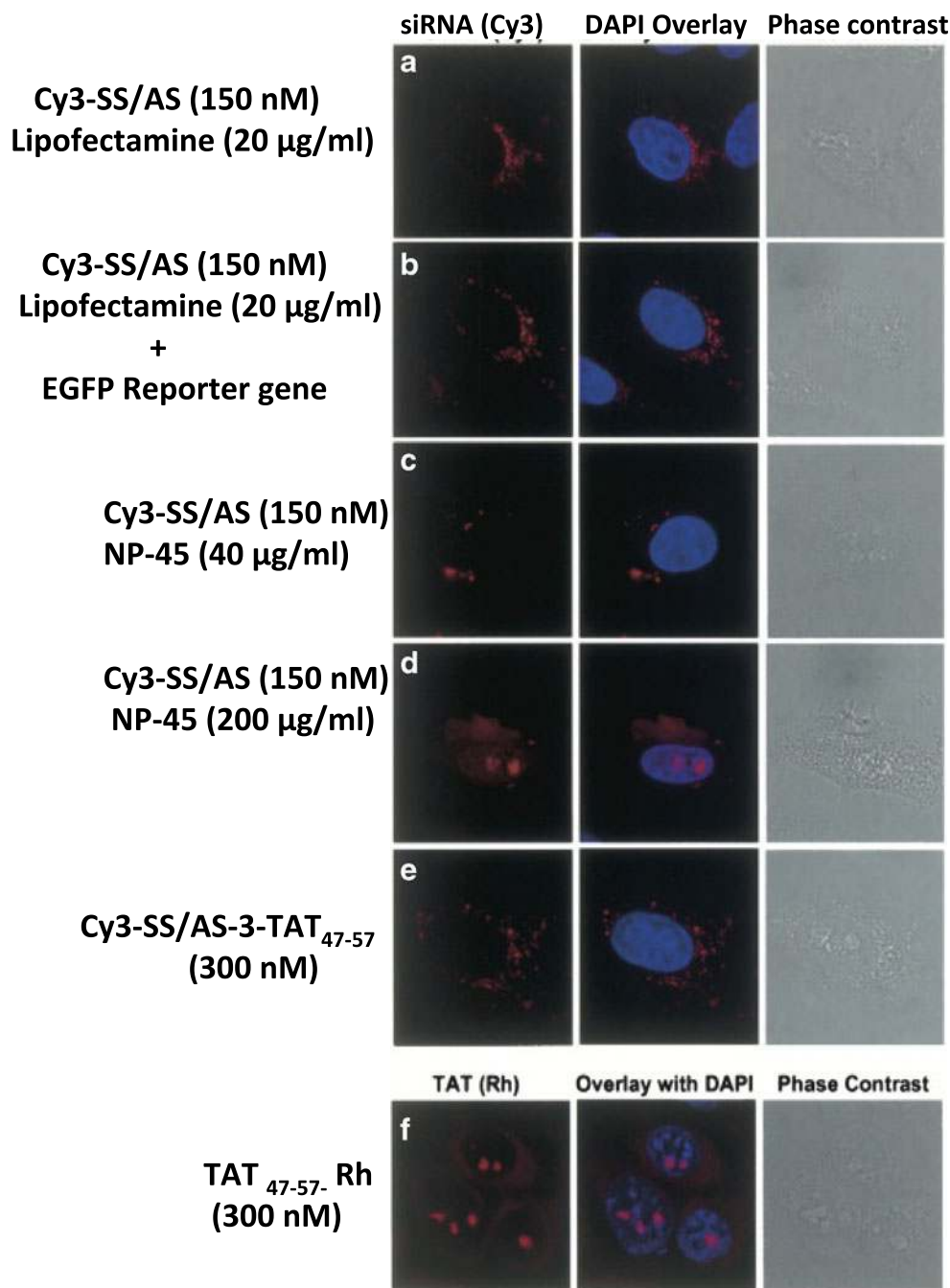
localization signal, this shuttling allows activated RISC to access its targets in cellular compartments like the nucleus (80). This mechanism may be responsible for the siRNA knockdown of small nuclear RNA (snRNA) 7SK observed by Berezhna et al.(79). RISC shuttling is assisted by Nuclear RNAi Defective (NRDE)-3, a cytoplasmic member of the Ago family, which functions as a transporter that can bind and help redistribute siRNAs to the nucleus. Nevertheless, the redistribution of NRDE-3 to the nuclear region is not possible in the absence of a nuclear localization signal (81).

Figure 1 outlines the key role of Exp5, a GTP dependent dsRNA binding enzyme as the cross point between the shRNA and miRNA nuclear export pathways. Owing to its saturability, Exp5 is the rate-limiting factor for these RNAi molecules (82). Interestingly, Ohrt and colleagues have shown that Exp5 is also involved in nuclear exclusion of microinjected siRNA molecules. Further, Exp5 knockdown resulted in accumulation of fluorescently labeled siRNa in the nucleus (83). If indeed Exp5 activity and siRNA silencing are interlinked, there is a possibility that that transfected siRNAs may compete with endogenous pre-miRNAs for binding to Exp5. This is feasible since si and miRNAs share features like dicer dependent processing and Ago-reliant gene silencing and the resulting competition will inhibit biological activity of endogenous miRNA similar to that resulting from overexpression of artificial short hairpin RNAs (shRNAs) (28,84). siRNA saturation of Exp5 may thus be a potential source of some off-target effects. These studies therefore not only stress the need for incorporating proper experimental controls in RNAi experiments, but also highlight the importance of using the lowest effective concentrations of gene silencing reagents to prevent the saturation of RNAi machinery.

Intracellular Processing and Nuclear Export of shRNA

shRNAs are synthesized as pre-shRNA by RNA polymerases II or III through promoters in the nucleus. The pre-shRNAis actively shunted to the cytoplasm by an Exp5 mechanism (82,85,86). In the nucleus, the shRNA hairpin structure binds GTP which, upon translocation to the cytoplasmic is hydrolyzed to GDP, releasing the loop-stem shRNA cargo (87). The pre-shRNA has its loop removed by Dicer-TRBP, yielding a 2 nucleotide overhang ds-siRNA (37,88). Microinjection studies in oocytes using adenoviral VA1 RNA have identified a 14 nucleotide or longer double stranded minihelical motif which is preferentially recognized by Exp5. RNA molecules containing this motif can bind to Exp5 for translocation to the nucleus. This RNA export motif has between 3–8 nts that protrude from the 3'end as well as a base-paired 5' end (89). This structural feature is also present in pre-miRNAs and is therefore

Fig. 3 Subcellular localization of siRNA was studied in HeLa cells. Cy3 labeled—siRNA was transfected by lipofectamine either alone (**a**), or with the reporter gene (**b**) into cultured cells (**a** and **b**), and nanoparticle NP-45 (**c** and **d**). Cy3-tagged EGFP siRNA was conjugated to TAT-domain for intracellular delivery (**e**) TAT peptide labeled with rhodamine as control (**f**) was also added to the cells. Alter 16 h, cells were fixed in methanol and siRNA duplex localization was observed by confocal microscopy. Reprinted with permission from Ref. (75).

siRNA (Cy3) DAPI Overlay Phase contrast

a Cy3-SS/AS (150 nM)
Lipofectamine (20 µg/ml)

b Cy3-SS/AS (150 nM)
Lipofectamine (20 µg/ml)
+
EGFP Reporter gene

c Cy3-SS/AS (150 nM)
NP-45 (40 µg/ml)

d Cy3-SS/AS (150 nM)
NP-45 (200 µg/ml)

e Cy3-SS/AS-3-TAT$_{47-57}$
(300 nM)

TAT (Rh) Overlay with DAPI Phase Contrast

f TAT$_{47-57-}$Rh
(300 nM)

incorporated when designing an artificial shRNAs in order for them to recruit Exp5 for efficient nuclear export. As shRNAs and pre-microRNAs are structural and functional homologs, overexpression of shRNAs can attenuate miRNA function by competing for Exp 5, which mediates the nuclear export of both these molecules. Further, this inhibitory effect could be overcome by overexpression of Exp5. For example, overexpression of Exp5 in human 293 T cells was shown to increase shRNA-mediated RNAi, suggesting the importance of Exp 5 in shRNA transport. (85).

As shRNA processing closely parallel the miRNA pathways, advances in understanding miRNA processing have also improved shRNA designs, especially the synthesis of shRNA with miRNA backbones (90,91). Thus there is ample scope for further optimizations of shRNA design to enhance Exp5 recruitment and improve the biological activity.

Recently, promising approaches have been shown to deliver external shRNA in an oral form. This involves the modification of live, non-pathogenic *E. coli* strains to express both shRNA as well as the protein invasin, which interacts with integrins on the epithelial cells to facilitate intracellular

uptake of delivery vehicle via an endosomal process. The subsequent release of shRNA within the cytoplasm of target cells is accomplished by the incorporation of pore forming protein listeriolysin O, which can selectively rupture the endosome and allow escape of shRNA. Once free in the cytoplasm of the epithelial cell, the shRNA is available for processing by the RNAi machinery to induce degradation (silencing) of β-catenin mRNA (Marina Biotech: http://www.marinabio.com/tkrnai_platform).

Kinetics of RNAi

RNAi in mammalian cells is effective for a relatively short duration of 5–7 days, principally due to its successive dilutions resulting from cellular division (92). The transient nature of mammalian RNAi is due to the lack of an amplification system similar to that found in *Drosophila*, where gene silencing can last many generations (93). For this reason, a single dose of siRNA may not decrease expression of proteins with long half-lives. Also, synthetic siRNA needs to be inserted in cells with the help of chemical agents that may affect their transfection efficiency and subsequent efficacy. The duration of gene silencing can be increased or decreased depending on siRNA backbone and the expression vector- plasmid or virus, used for shRNA delivery, with the latter being significantly more effective owing to the difficulty associated in transfecting plasmids. The gene silencing effected by siRNA can be increased by backbone modifications and for shRNA by using a lentiviral vector. Overall however, shRNA is capable of longer lasting and more stable gene silencing compared to synthetic siRNA. We have demonstrated very short gene silencing of caspase-3 lasting upto 2 days when pancreatic β-cells were transfected with siRNA/lipofect-amine-2000 complex. In contrast, transduction with ade-noviral vector encoding caspse-3 shRNA (Adv-caspase-3-shRNA) showed gene silencing at least up to five days. Further, when human islets transduced with Adv-caspase-3-shRNA were transplanted in diabetic immunodeficient mice, they remained viable and restored normoglycemia for at least two weeks within one day of transplantation (Fig. 4)(94). Since the viral vectors used in this study were replication deficient, long-lasting gene silencing was obtained without any side-effects, suggesting the impor-tance of utilizing the right delivery vehicle for *in vivo* gene silencing by shRNA.

Several factors affect the duration of gene expression silencing. Some of these are the time it takes to undergo cell division, siRNA treatment regimen, and the method used to deliver siRNA. In the recent years, studies to understand the kinetics of the intracellular RNAi process have been carried out. These include mathematical model of RNAi-mediated gene silencing by Bergstrom and co-workers and Groenenboom and colleagues to understand mechanism of gene silencing due to transgene or virus (95,96). Raab and Stephanopoulos examined the dynamics of dose- and time dependent gene silencing by siRNA relative to plasmid transfection (97). Bartlett and Davis have combined intracellular imaging with mathematical modeling to trace siRNA induced RNAi from initial delivery to final intracellular function. Their studies on silencing of Lucifer-ase gene confirmed their hypothesis that siRNA-dilutions due to cell division are the major factor controlling the duration of gene knockdown in dividing cells. In this study gene silencing in nondividing cells lasted three times as long compared to dividing cells (3 weeks *vs.* 1 week) both *in vitro* and *in vivo* (98). It was also shown that gene silencing could be maintained for longer periods in slow-dividing cells, thus confirming that the dilution effect due to cell doubling time may be responsible for the less durable silencing in rapidly dividing cells. This is in agreement with previously reported kinetics of siRNA-mediated gene silencing in non-dividing and differentiated cells, such as neurons and macrophages. Gene silencing lasting for upto 3 weeks was observed in neurons transfected with a synthetic 21-nt siRNA, while RNAi by the same siRNA was lost after five days in proliferating cells. This suggests that not only is RISC containing the siRNA duplex more stable in non-dividing cells, but the actual number of RISC in such cells may be constant (99). Studies in macrophages infected experimen-tally with human immunodeficiency virus (HIV) have suggested that the persistence of RNAi is dependent on the presence of the target RNA as pretreatment with siRNA targeting endogenous gene silenced gene expression up to 15 days (100). Thus, siRNA-mediated RNAi may be more effective in non-dividing cells than in proliferating cells. Since both double-stranded and single-stranded siRNAs are attacked by serum nucleases, enhancing their stability would be expected to increase the duration of gene silencing by increasing their intracellular persistence. Layzer and colleagues have used chemically altered siRNA to study gene silencing in HeLa cells (101). Their results indicate that increasing the serum stability of siRNAs by chemical modifications had no corresponding effect on the duration of gene silencing. This suggests that in fast dividing cells the dilution effect due to cell division rather than siRNA stability is the rate limiting step in the duration of gene silencing. Recent work investigating the duration of silencing by siRNAs points to the nature of the 3'-overhang nucleotides of the guide strand of siRNAs as a major factor. While the presence of deoxyribonucleotides in overhangs has a negative impact on the duration of RNAi, ribonu-cleotides or chemically modified nucleotides can increase the duration of RNAi. Further, the siRNA sequence also determines the duration of silencing of siRNAs (102). The relative persistence of naked siRNA in non-dividing cells
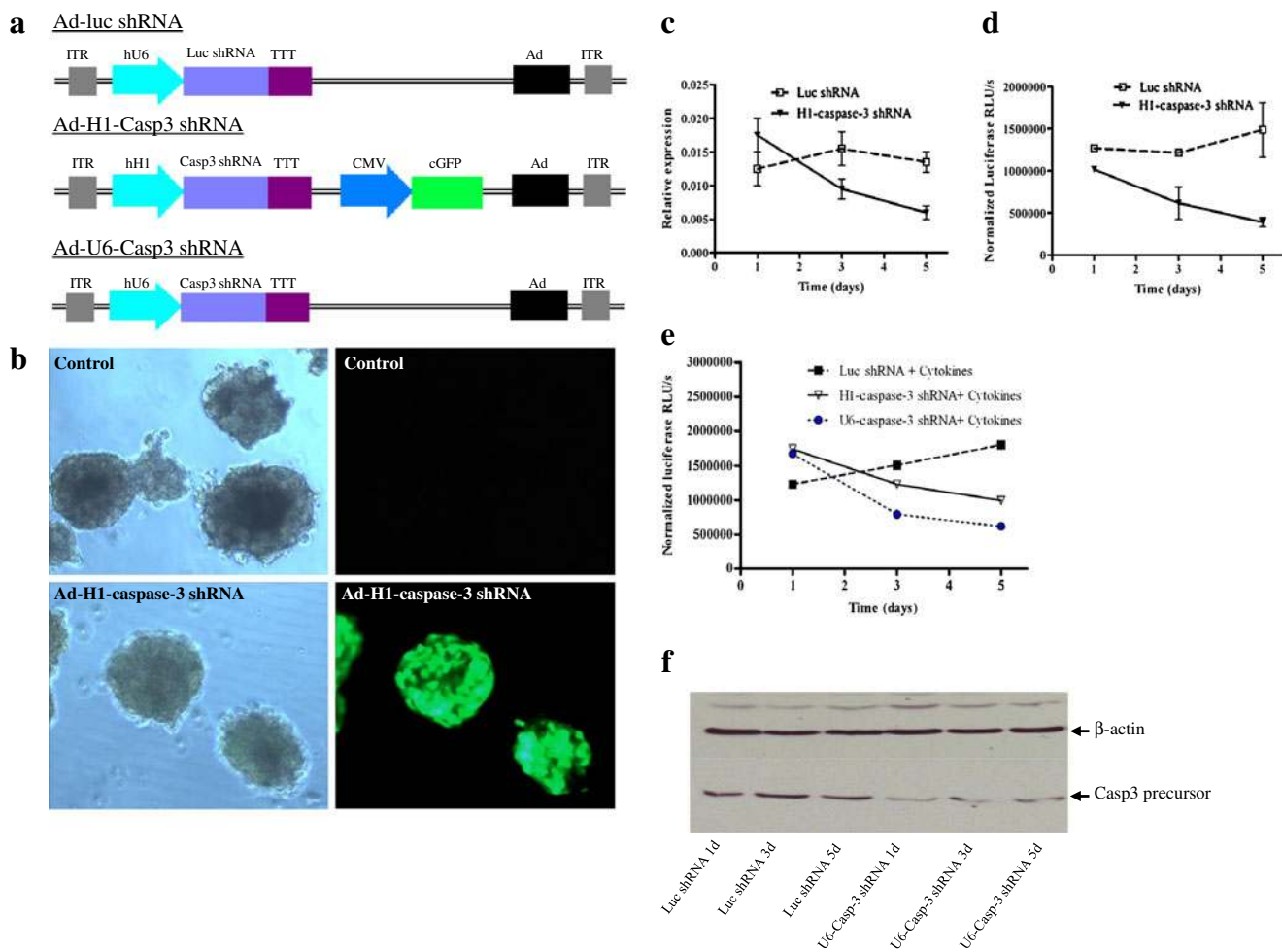
Fig. 4 Recombinant adenoviruses (Adv) encoding shRNAs targeting caspase-3 gene under different promoters caused RNAi in human islets and blocked apoptosis. (**a**) Scheme for preparing recombinant adenovirus encoding shRNAs. Luciferase shRNA was used as control; Adv-H1-caspase-3-shRNA expressed caspase-3 shRNA under a H1 promoter; Ad-U6-caspase-3 shRNA was driven by a U6 promoter. (**b**) The transduction efficiency of Adv-shRNA in human islets was observed 3 days post-infection with caspase-3 shRNA carrying vector under a fluorescent microscope with un-infected islets as control. (**c**) Adv-H1-caspase-3- shRNA was used for transducing human islets and transcriptional activity observed at the indicated days. (**d**) Effect of Adv-H1-caspase-3-shRNA on gene silencing was assessed by measuring caspase 3/7 activity in isolated human islets. (**e**) Comparison of different promoters driven shRNAs silencing effect on caspase activity induced by cytokine cocktail. After transduction with Adv-U6-Caspase 3 shRNA, Adv-H1-Caspase-3-shRNA and Ad-luc shRNA, the infected and control islets were incubated with the fresh medium containing the cytokine cocktail, then were collected at the indicated days for determining caspase activity. (**f**) Evaluation of Adv-U6-Caspase-3 shRNA silencing effect by Western blotting. Reprinted with permission from Ref. (94).

observed by Bartlett and Davis may be a result of stability of the single-stranded guide strand incorporated into RISC (98). Double-stranded RNA is less sensitive to the action of nucleases compared to single stranded RNA (103). Hoerter and colleagues have demonstrated that 21- and 24-nucleotide double-stranded RNAs are more stable in human cytosolic cell extract than in serum, whereas smaller (18-nucleotide) double-stranded RNA was rapidly degraded in both fluids. Their results suggest that cellular environment may be protecting molecules involved in RNAi. Some of the protection offered to 21 and 24 nt RNA was also due to their efficient incorporation into RISC-like structures while 18-mer dsRNA molecules were inefficient in incor-

porating (104). This may help to explain the intracellular persistence of non-modified naked siRNA molecules in previously discussed reports.

A typical siRNA dosing is effective for a maximum of $3 \sim 7$ days (105). However, many clinical siRNA treatments may require gene silencing for longer time periods. Efforts have been made to increase the duration of gene silencing by delivering shRNAs through lentiviral vectors which causes stable and persistent gene silencing, in studies carried out both *in vitro* and *in vivo* (106–109). To attain clinical translatability, controlling intracellular siRNA levels, including a 'turn off' when not needed is a major challenge to shRNA application. As siRNAs are by nature not long

lasting, they are useful in treating pathologies where only short duration gene silencing is needed.

Several important design yardsticks have to be considered when evaluating the efficacy of siRNA against certain genes coding for proteins with long half-lives. For proteins with long half-lives (> 48 h), it makes sense to observe the knockdown phenotype at later time points rather than commonly used early time points (48–72 h) since the silencing phenotype will be visible only after the protein levels drop below a threshold (98). Also, the parameters inherent to the target cell such as cell doubling time (accounts for dilution effect), compartment volume (controls the siRNA concentration), and the half-life of the target transcripts and proteins need to be factored into the calculations. The ability to calibrate the levels of gene or gene product silencing will go a long way in improving the therapeutic value of RNAi in a clinical setting.

OFF-TARGET EFFECTS

Off-target effects are the gene perturbations caused by unintended interactions between the RNAi molecules and cellular components. First reported by Jackson and colleagues in 2003 (27), off target effects can complicate the interpretation of RNAi data (110). Broadly speaking, off-target effects can be specific or non-specific. The former are caused by the limited degree of siRNA or shRNA complementarity to non-targeted mRNAs. The latter, which causes immune and toxicity related response, are due to the RNAi construct itself or due to the delivery vehicle transporting the siRNA. The delivery associated off-target effects can be subsequently divided into three separate categories. One, the miRNA-like off-target effects which result from partial sequence homology to the 3′ UTRs, two, siRNA/delivery vehicle may trigger an immune response by activating Toll-like receptors (TLR); and three, the endogenous RNAi machinery may get overwhelmed (saturation effect) due to the introduction of RNAi molecules, impacting miRNA processing and function (111,112).

Specific or miRNA-Like Off-Target Effects

Specific silencing of unintended targets results from an imperfect hybridization between the guide strand of the RNAi molecules with non-target transcripts. As this type of complementarity is a characteristic of miRNA process, hence the term. A gene expression microarray analysis by Jackson *et al.* (2003) revealed that the off-target expression patterns were independent of siRNA concentration and were constant across the cell types tested. The involved genes had sequence similarity of about 11 nucleotides with

the 3'end of siRNA sense strand. It was therefore concluded that the unique and reproducible gene expression pattern observed, suggested a siRNA-specific effect (27). RNAi-inducing constructs possessing complementarity in the seed hexamers are capable of similar off target gene expression, irrespective of cell type and of the delivery vehicle being used. Interestingly, even sub-optimal (<7 nucleotides) sequence complementarity of siRNA guide sequence with the targeted mRNA, can suppress non-targeted genes. It is now understood that for off-targeting mechanism to occur, a perfect complementarity between nucleotide position 2–7 or 2–8 (seed region) of the antisense strand and the 3'UTR of the transcript is necessary (111,113).

While the 'seed region' sequence may be necessary for off-targeting, it cannot trigger the off-target effects by itself. Nielsen and colleagues have reported that, the presence of adenosine opposite to siRNA base 1 and of an adenosine (A) or uridine (U) opposite to siRNA base 9 enhanced off-target mRNA repression. They also discovered that specific sequences within a few bases of the 5' and 3' of the seed region, as well as increased AU content in the 3' of the seed region could independently enhance off-targeted down regulation of the transcript (114). Both sense and antisense strand are capable of triggering off-target effects, particularly if the sense strand is preferentially up-taken by RISC. This can occur even though RISC has differential affinity for these two siRNA strands. It has been demonstrated that while both sense and antisense strand could be taken up by RISC, the efficiency of target degradation by the complex was highly dependent on the ability of each strand to be assembled onto RISC, which in turn was directly related to its base sequence (27,115). Other relevant study in this respect demonstrated that while there was significant correlation between *in vitro* and *in vivo* RNAi activity within the species, there was interspecies variation in off-target effects. This has been attributed to sequence differences in the 3'UTR region of various species and consequently, there is less chance of successfully predicting human off-target effects using a murine model (116).

RNAi regulation by miRNAs involves a partial complementarity between the target RNA and the miRNA. As miRNAs cause gene silencing through mRNA degradation and translation inhibition, the siRNA mediated off-target effects may be acting at two levels as well. For this reason, greater emphasis needs to be paid to not only improve siRNA design, but also monitoring the gene and protein levels following RNAi therapy to factor in any off-target effects.

Non-specific Off-Target Effects

Nonspecific off-target effects include immune system activation in response to exogenous gene silencing molecules, cellular toxicity due to the composition of the RNAi molecule,

and various effects caused by the chemical composition and nature of the delivery vehicle. The off-target effects of shRNA and siRNA are different owing to the fundamental differences between these two RNAi approaches. For example, since shRNA is expressed in the nucleus and processed by the endogenous machinery, it is less likely to trigger an immune response *in vivo*. Also, the 5′ ends of such endogenously spliced shRNA oligomers are less inflammatory than the 5′ ends of exogenous siRNA molecules (117–119). It was seen that synthetic siRNA delivered to primary hematopoietic cells induced upregulated type I IFN genes and increased IFN-synthesis. In contrast, endogenously expressed shRNAs bearing the same sequence could not generate an immune response. The immune system activation in off-target effects is similar to that activated following viral infection (120) with siRNA and siRNA sequence motifs stimulating the innate immune response (121).

The primary immune trigger in mammalian cells is the introduction of 29–30 bp or longer dsRNA. Activation of the innate immune response causes upregulation of IFN-stimulated gene expression on one hand and leads to global degradation of mRNA and inhibits of general protein translation. It should be noted that the simultaneous transfection of both synthetic siRNA oligomers and shRNA can generate an interferon response which may have a sequence dependency and, can be thus circumvented by improved design parameters. Upon transfection of synthetic/exogenous RNAi to the cell, immune activation involves cytoplasmic and endosomal mechanisms. Prominent endosomal mediators of nucleic acid stimulated immune response are Toll like receptors (TLRs), a family of cell surface receptors primarily involved in identifying pathogen though their associated molecular patterns (122). TLRs 7 and 8 are modulated by single stranded RNA, TLR 9 by unmethylated CpG (usually found in bacteria) and TLR 3 is activated via dsRNA transfection (122–125). In some cases, immune stimulation rather than the targeted effect may be behind the observed therapeutic effects of siRNA. Kleinman and colleagues (2008) used a variety of 21 nt longer siRNAs that targeted, in addition to *VEGFA* and *FLT1,* a variety of non-specific as well as non-expressed genes, as well as pro- and anti-angiogenic genes (26). It was shown that all sequences 21 nts or longer (but >19 nt), similarly suppressed choroidal neovascularization (CNv) in human patients with age-related macular degeneration (AMD). The siRNA effect was found to be mediated by activation of TLR3 which caused the release of IFN-γ and IL-12 resulting in nonspecific anti-angiogenic effects (26). To summarize, these studies indicate that immune activation by siRNAs can adversely impact the clinical outcomes and affect the health of the recipient. In mice, these immune responses can cause organ toxicity and decrease the numbers of lymphocytes and platelets (126). Another

study reported the proliferation of breast cancer cells in response to treatment by both gene-specific and control siRNAs (127). Taken together, there is a need for improved siRNA design, establishing experimental controls and careful interpretation of the resulting data.

Other non-specific off-target effects are due to the saturation of RNAi machinery, especially Exp5, in the cell. Blockade of cellular sh/miRNA pathways can lead to liver toxicity and fatal effects in mice in a dose-dependent manner (128). Similar liver toxicity and morbidity were seen in another study where RNAi was used to knockdown multidrug transporters. Positive results were obtained when artificial miRNA were used, suggesting that overexpression of shRNA impairs miRNA processing (129). As expected, Exp5 overexpression enhances RNAi mediated by shRNAs and miRNAs by eliminating the export chokepoint and making cells tolerate higher amounts of shRNA (84). Further, synthetic siRNAs can displace endogenous miRNA from RLC, leading to off-target de-repression of multiple mRNAs (130). Even though synthetic siRNAs may not require processing or export from the nucleus, they can saturate some components of the cellular RNAi machinery, suggesting that cells have a limited capacity to assemble the RISC complex on exogenous siRNAs, and this might affect miRNA expression levels and function. Transcripts with seed sites for highly-expressed endogenous miRNAs were significantly upregulated in cells transfected with synthetic siRNAs or miRNAs. Targets containing sites for other endogenous miRNAs, in addition to the transfected miRNA, exhibited reduced silencing, suggesting the competition of the transfected miRNA with the endogenous miRNAs. Consequently, both intended and off-targeted gene silencing by synthetic siRNA increased with dose. An endogenously expressed shRNA controlled by a Pol II promoter and constructed in an adenoassociated virus silenced its target for at least one year after a high initial dose (131). Transfection of shRNA in cells has allowed measurement of the extent to which endogenously produced miRNA is downregulated due to the competition for Exp5 and Ago-2. These studies indicate that while more information is needed before strategies to overcome RNAi machinery saturation and shRNA toxicity can be standardized, the choice of promoter used (incorporating pol II instead of pol III) and keeping the amount of shRNA dose well below the point at which it competes with endogenous miRNA can ameliorate this type of non-specific off-target effects.

STRATEGY FOR OPTIMAL GENE SILENCING

Systemic delivery of siRNA is required for treating conditions like metastatic tumor. Degradation by RNAses in the plasma

reduces the efficacy of systemic RNAi. Treatment efficiency is further reduced if there is a lack of targeting ability. Finally, the siRNA as well as the delivery system may cause the activation of immune system. Several delivery strategies have been proposed over the years.

An ideal delivery system can reversibly bind siRNAs so that after delivery, it can be easily released in the cytoplasm of target cells and also protects siRNAs from nuclease mediated degradation during its passage through the circulatory system. It should allow escape from endosomal compartment and should be non-toxic. Finally, this system should not be cleared too quickly by the kidney. In this section, we discuss various approaches for improving the overall *in vivo* siRNA targeted delivery and reduce off-target effects.

Design Modification

The extent of gene silencing depends on the siRNA sequence. Even minor changes in sequence can alter gene silencing efficiency dramatically. For example, sequence alterations by mutation in the antisense strand may result in non-recognition of the homologous mRNA by the loaded RISC. The design parameters that determine the effectiveness of RNAi includes thermodynamic stability, the number of nucleotides in the siRNA sequence the% of GC residues, the secondary structure of the RNA, the number of sites with single nucleotide polymorphism (SNP) and the number of repeats (132). The use of commonly available computational design tools allows the synthesis of potentially effective siRNA sequences. As explained earlier, the rules for designing siRNA and shRNA sequences are similar since shRNA ultimately causes RNAi by generating a siRNA. For this reason, siRNA designing software can be used to design shRNA sequence as well. There are some differences however. In addition, for efficient shRNA expression, presence of GC at position 11 and AU at position 9 is highly desirable (133). In certain cases, especially where the siRNA software does not yield efficient shRNAs (134), a number of sequences need to be generated and tested to find the most efficient shRNA. This can be highly challenging due to the time and effort it takes in cloning shRNA into plasmid. This issue is circumvented by purchasing several commercial pre-designed shRNA plasmids and screening them.

The extent, length, and efficiency of shRNA-mediated gene silencing depend not only upon shRNA sequence design but a proper shRNA expression cassette as well. In addition, the manipulations of shRNA backbone can impact its processing and export. One way to improve shRNA efficiency is to insert a miRNA backbone while designing shRNA. The use of miRNA backbone makes shRNA less cytotoxic (135) and increases the specificity of gene silencing in a tissue or cell specific fashion (114). Boden and colleagues synthesized a shRNA with a miR-30 backbone against HIV-1 trans-activator protein TAT (136). This was more potent in causing RNAi than a conventional shRNA without miR-30 incorporation. However, Li and colleagues showed opposite results when they compared conventional and miR-30 based shRNA against the transgene luciferase and endogenous mouse tyrosinase gene (137). Their findings indicate that shRNA based constructs were significantly better at gene knockdown than miR30-based constructs. Han and colleagues embedded a pol II controlled shRNA in a miRNA backbone and compared the silencing of alpha-synuclein (SNCA) gene in 293 T cells. While the extent of silencing by both constructs was similar, the vector with miR-30 backbone was less cytotoxic than conventional shRNA, suggesting that the miRNA-embedding approach may be more effective *in vivo* in some cases (138).

Our recent incorporation of miRNA backbone in shRNA has effectively caused TGF-β1 gene silencing in a specific manner by reducing both the mRNA transcript and down-regulating protein expression. The vectors used had a pri-miRNA construct and shRNA expression was under the control of a glial fibrillary acidic protein (GFAP) promoter for reducing off-targeted TGF-β1 gene (139). In another study, our bipartite plasmid vectors co-expressed a vascular endothelial growth factor (VEGF) cDNA and incorporated shRNA to target the inducible nitric oxide synthase (iNOS) gene (Fig. 5). As can be seen, cDNA insertion downstream of the CMV promoter and upstream of miR30 caused nearly 60% reduction in nitric oxide production compared to only 40% observed with miR30 shRNA alone. We have hypothesized that increasing the distance between CMV promoter and miR30 shRNA may allow for optimal gene silencing. We also tested the efficacy of gene silencing when a variety of promoters- H1, U6 and cytomegalovirus (CMV) as well as and miRNA backbones were incorporated. While there was no difference in gene silencing in presence of different promoters, insertion of miR30 backbone into human iNOS shRNA was actually less effective than conventional shRNA (140). Taken together, these results indicate the need for further studying shRNA design parameters to develop clinically competent shRNAs as well as the need for standardizing assay conditions while measuring the effect of backbone alterations in sh/miRNA-based RNAi.

Backbone and Structural Modification

Even though siRNA duplexes used for silencing are inherently stable, chemical modification can improve serum stability and reduce off-target effects, while helping target the siRNAs into cells and tissues. The backbone modification includes incorporation of phosphodiester or ribose units. Modifications of RNA at the 2'-position of the ribose ring
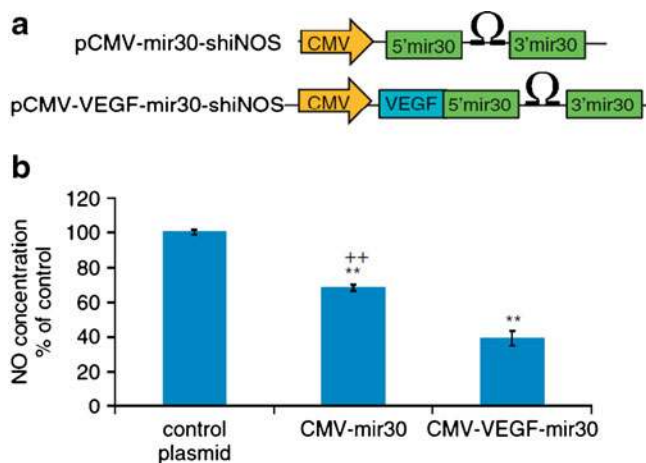
Fig. 5 Insertion of VEGF cDNA upstream of mir30-shRNA enhances silencing of the iNOS gene. (**a**) Schematic of pCMV-mir30-shiNOS and pCMV-VEGFmir30-shiNOS design. (**b**) AD293 cells were transfected with pCMV-mir30-shiNOS, pCMV-VEGF-mir30-shiNOS and control plasmid. Production of nitric oxide was assessed using Griess assay. Results are the mean ± SE of four experiments. $p < 0.01$ compared to the control plasmid group; ++ $p < 0.01$ compared to the pCMV-VEGF-mir30 group. Reprinted with permission from Ref. (140).

increase siRNA stability by denying the action site to endonucleases and reducing immune response activation. The resulting molecule is resistant to nucleases and has an increased half-life *in vivo* (141,142). Apart from modifying the chemical bond, the sugar unit may also be modified as in 2′-fluoro (2′-F), 2′-O-fluoro-β-D-arabinonucleotide (FANA) and 2′-O-(2-methoxyethyl) (MOE) as well as during synthesis of modified RNA nucleotides termed locked nucleic acid (LNA).

Chemically modified siRNAs are resistant to nuclease degradation and demonstrate improved pharmacokinetics and biological activity with a concomitant decrease in nonspecific targeting. Many of these new siRNA modification approaches rely on the experience gained by previously studied backbone modifications of antisense ODNs (102). In addition, 2-O-methyl modification in the seed hexamers of the guide strand can reduce off-target effects. When combined with simultaneous insertion of 2-O-methyl group in the passenger strand, efficient RNAi was observed without any off-target silencing (111). 2-O-methyl alterations of specific U or G residues in the passenger strand reduced immune activation but had no negative effects on RNAi (122). Insertion of chemical modifications is an important strategy to improve gene silencing and reduce non-specific off-target effects.

Complexation

RNAi molecules carry a net negative charge which allows them to form stable complexes with positively charged

carriers. The resulting complex can easily move across the plasma membrane to reach the cytoplasm. Several such cationic lipids are commercially available for laboratory research. These include agents like Fugene HD, lipofectamine, and oligofectamine. We have synthesized a cationic lipid bearing a methylsulfonic acid tag in the cationic head to increase the extent of lipid/DNA interaction. Our studies in intact human islets have shown that higher siRNA transfection efficiencies could be obtained by this delivery vehicle than by lipofectamine (143). Using a similar strategy, Zimmermann and colleagues silenced apolipoprotein (ApoB) expression in monkeys for upto 11 days following i.v. injection of siRNA/lipid complexes. Others have formulated siRNA with anisamide-conjugated poly(ethylene glycol) (PEG) to demonstrate significant gene silencing following systemic dosing in a lung metastasis mouse model (144). Neutral phospholipid-based liposomes coated with hyaluronan and carrying cyclin D1 (CyD1) siRNA (145) have also been used for systemic siRNA delivery. Biodegradable cationic polymers that dissociate siRNA from polycationic carrier in the endosomes can deliver siRNA efficiently with low toxicity. These contain disulfide bonds which keep the complex intact before cellular uptake. Subsequently, in the reducing intracellular environment of the cytosol, the disulfide bonds are cleaved, and the siRNAs released (146–151). One of the major barriers in the nucleic acid delivery is the lack of endosomal release. Therefore, attaching 'endosomal escaping' chemical units like poly-histidine (152), and other protonatable amino acids as pendant groups (150) has a positive effect on RNAi.

Cell penetrating peptides (CPPs) are short length (<30 amino acids) cationic polypeptides which use electrostatic attractions to bind negatively charged cell surface, thereby getting internalized through endocytosis mediated process, along with any attached cargo, including siRNA. siRNA cargo attachment strategies to CPPs such as TAT, penetratin, and Transportan has been reviewed by Endoh *et al.* (153). Following cellular internalization, the si/shRNA/peptide complex releases the RNAi molecule (154). However, the utility of CPPs may be reduced owing to their entrapment following endocytosis. For this reason, developing CPPs that promote endosomal escape is a prerequisite for successful siRNA strategy. Fusogenic peptides are synthesized to mimic the fusion domain sequence of the influenza virus which allows it to escape from endosomes to cytoplasm. Fusogenic peptides and lipids can facilitate siRNA release into the cytoplasm from the endosomes. Recently, CPPs conjugated to steric acid units and covalently attached pH sensitive moieties were shown to facilitate endosomal release. Strong RNAi responses in primary cells and in different murine organs following systemic delivery in mice were also noticed without any associated toxicity (155).

Incorporation of polypeptides derived from the endodomain of the influenza virus fusogenic peptide (diINF-7)

significantly promoted the liposomal fusion with the endosomal membrane, enhancing siRNA escape into the cytoplasm (156). A nanocarrier containing octaarginine (R8) for tumor targeting was reported by Sakurai and colleagues. Here, the siRNA is encapsulated within a biodegradable polyethylene glycol (PEG)-lipid which carries a pH-sensitive fusogenic peptide for endosomal release (157). Dioleoyl phosphatidylethanolamine (DOPE) is a fusogenic lipids and can ensure efficient endosomal release of siRNA cargo following systemic treatment. In addition, the specially designed fusogenic peptide carrier will be preferentially cleaved in the matrix metalloproteinase (MMP)-rich environment of tumors, thereby ensuring minimal off-target effects. Highly efficient siRNA-mediated luciferase silencing in fibrosarcoma cells *in vitro* and xenograft tumors using this method was also described (158). Stimuli-triggered macromolecule release from the mildly acidic endosome (*e.g.* pH 5.0–6.0) has also been investigated. Recently, Shim and Kwon have used a non-cytotoxic, polyspermine gene carrier to deliver both plasmid DNA and siRNA *in vivo*. This carrier is designed to degrade differentially in the endosome and the cytosol (159).

Polyethylenimine (PEI) is a synthetic polymer capable of forming non-covalent complexes with siRNAs and protecting them from nucleolytic degradation. siRNA complexed with PEI can be efficiently taken up via endocytosis and released through the 'proton sponge effect' in the cytoplasm (160). Arginine-glycine-aspartate peptides (RGD) are known to traverse through the plasma membrane passively and localize to the cytoplasm, making them an efficient siRNA carrier. PEI-g-PEG-RGD linked to VEGFR1 siRNA through a PEG spacer was systemically delivered to solid murine tumors, resulting in angiogenesis inhibition in tumor (161). RGD peptide-labeled with chitosan nanoparticle was also reported to be effective in tumor targeted delivery and silencing multiple growth-promoting genes in tumor bearing mice (162).

The conclusions of the first clinical trial using systemically delivered siRNA demonstrating RNAi for solid tumors have been reported by Davis and colleagues (163). Using a targeted delivery system in a human clinical trial, the authors demonstrated intracellular nanoparticles in dose-related amounts and the reduction of target mRNA and protein in tumor biopsies. In addition, a specific mRNA fragment was detected, indicating the intended site-specific siRNA-mediated mRNA cleavage. These nanoparticles consist of four components – a cyclodextrin-based polymer (CDP), a targeting ligand against the human transferrin (hTf) protein, siRNA targeting the ribonucleotide reductase enzyme, and PEGylation for enhanced plasma retention. In this system, the siRNA is complexed inside the CDP which has encapsulating cavity exposed to the outside and the PEG molecule is conjugated with adamantane (AD) such that AD-PEG forms inclusion complexes with surface-exposed CDs. The hTf targeting ligand bound to AD-PEG chains at the surface exposed end of the PEG forming AD-PEG-hTf.

Bioconjugation

Bioconjugating siRNA strands with lipid units or biodegradable polymers can enhance the efficiency of siRNA delivery and uptake. This can increase the thermodynamic and nuclease stability of siRNA and improve their pharmacokinetic profile. Bioconjugation also enables the targeting of siRNA to specific cell types. Conjugation of ODNs with cholesterol increases the net hydrophobicity allowing easy passage through lipid bilayer and increased cellular uptake and hepatic deposition after i.v. injections (15). We have also shown that ODNs can be conjugated to galactose-PEG to increase stability and ensure targeted delivery to hepatocytes *in vivo*. The presence of an acid-sensitive ester linker ensures cleavage and release of free ODN in the acidic environment in endosomes (164).

We have recently shown that the conjugation of sense strands of TGF-β1 siRNA to galactosylated poly(ethylene glycol) (Gal-PEG) and mannose 6-phosphate poly(ethylene glycol) (M6P-PEG) via an acid labile ester linker resulted in the targeted delivery of siRNA to HepG2 cells expressing asialyoglycoprotein receptors and immortalized hepatic stellate cells (HSC-T6s) expressing M6P-insulin growth factor II (M6P-IGFII), respectively (165). Even in the absence of cationic carrier, almost 40% gene silencing was observed at a dose of 400 nM M6P- and Gal-conjugated TGF-β1 siRNA (Fig. 6). Significantly, non-conjugated siRNA caused no gene silencing at a similar dose. This method offers significant advantages over using cationic lipids as gene carriers as the latter may bind to negatively charged serum protein and form aggregates. This will not only alter the biodistribution profile but also induce release of inflammatory cytokines and activate complementary system, resulting in off-target effects. We are currently in the process of testing these formulations *in vivo*.

Conjugated siRNA uptake is usually via receptor-mediated endocytosis. In addition, there is enhanced permeability for the conjugate due to increased hydrophobicity and charge. By conjugating cholesterol to the sense strand of siRNA, Soutschek and colleagues observed to effective silencing ApoB gene after i.v. administration in mice (166). Cholesterol conjugation is also used to generate antagomiRs (single strand ODNs that are cholesterol-linked at their 3′end). These molecules have the ability to silence endogenous miRNA by a process termed as 'antagomirization' (56). Upon insertion into the cell, they are irreversibly bound in a perfectly complementary manner to the specific
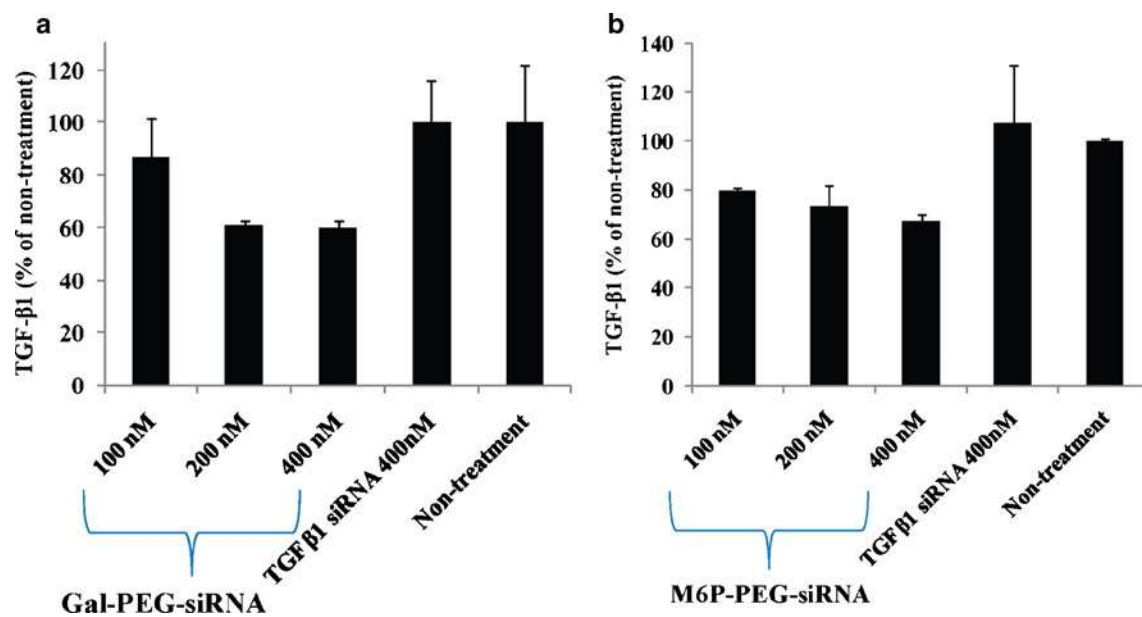
Fig. 6 Effect of Bioconjugation on Gene Silencing (**a**) Naked or Gal-PEG conjugated siRNAs against TGF-β1 gene were added to cultured HepG2 cells. (**b**) Naked or M6P-PEG conjugated siRNAs were added to cultured HSC-T6 cells. No cationic lipids were used to facilitate siRNA uptake. Following transfection, the medium was replaced and cells incubated for another 42 h. Bioconjugation of siRNAs with Gal increased their targeted uptake by hepatocytes, while M6P conjugation targeted siRNAs for increased uptake by hepatic stellate cells (HSCs). Gal: galactose; PEG: galactosylated poly (ethylene glycol); *M6P-PEG*: mannose 6-phosphate. Reprinted with permission from Ref. (165).

miRNA target resulting in the inhibition of Ago2 cleavage. AntagomiRs can constitutively inhibit the activity of specific miRNAs. The antagomirization of miR 133 resulted in cardiac hypertrophy (167). Aptamers are defined as a single-stranded RNA or DNA ODN ranging in length from 15 to 60 bases that bind specifically to specific molecular targets. Anti-gp120 RNA aptamers were covalently conjugated with one strand of siRNA and the other strand was annealed to the former strand. These aptamer–siRNA conjugates were able to enter HIV-infected cultured cells and silence viral replication (168).

Antibodies can serve as effective targeting ligands when the targeted antigen is exclusively expressed or substantially overexpressed on target cells. Examples of such bioconjugates include a HER-2 siRNA-carrying liposomes tagged with transferrin receptor-specific antibody fragments (*i.e.* nanoimmunoliposomes) that silenced the HER-2 gene in xenograft tumors in mice, and inhibited tumor growth (169). A liposomal siRNA carrier system decorated with an anti-CD33 antibody fragment effectively targeted and delivered siRNA to CD33+ acute myeloid leukemia (AML) cells (170). Monoclonal antibodies targeted against specific cell types can also be used to deliver siRNAs enclosed in liposomes. A recent study has utilized humanized Lewis-Y monoclonal antibody to selectively deliver STAT3 siRNA to Lewis-Y protein expressing cancer cells via antigen recognition (171). Pirollo and colleagues employed cationic immunoliposomes decorated with anti-transferrin receptor antibody. The resulting immunolipo-

somes delivered siRNAs efficiently after systemic administration to primary and metastatic prostrate, pancreas and breast cancers (172). Folic acid (FA) is needed for rapid cell growth, and many cancer cells overexpress folate receptors which are druggable targets (173,174). FA can be easily conjugated onto the surface of liposomal and polymeric siRNA carriers (175). FR-targeting, drug carrying nanoparticles have been shown to overcome drug resistance in P-glycoprotein (P-gp)-overexpressing human cancer model (176). Further, FR-beta-specific human monoclonal antibody can be used to treat rheumatoid arthritis as well as FRβ positive cancers (177).

CONCLUDING REMARKS

A number of challenges have to be met before RNAi can become a standard therapy. These include barriers to systemic delivery of RNAi molecules and off-target effects that can lead to unwanted or unexpected toxicities. Targeted delivery of siRNA/shRNA can greatly facilitate widespread therapeutic application of RNAi. Also, targeted distribution in the cell is important for optimal function. Apart from the barriers to systemic delivery, cellular uptake and intracellular trafficking remains a major barrier for RNAi molecules. The extent of these limitations varies among si-, sh- and miRNA. Unlike siRNA which is targeted to cytoplasm, shRNA expression vector must translocate into the nucleus for transcription into shRNA, which requires nuclear export to cytoplasm and

subsequent conversion to siRNA. Thus, cellular trafficking remains a major barrier, especially for shRNA and externally added miRNAs. Despite this limitation, shRNA is promising option since it provides persistent and stable gene silencing if viral vectors are used. In addition, they can be produced in large quantities.

While some advances have been made in understanding the saturation kinetics of endogenous RNAi processing machinery by si/shRNA, little is known about potential rescue tools. For this reason, it is essential to understand the mechanism through which siRNAs compete with endogenously produced miRNAs. The problem is further complicated by recent findings that while there is correlation between *in vitro* and *in vivo* data within same species, it may not be possible to extrapolate the finding about off-target effects from pre-clinical rodent models to predict the effects on humans. Significant advances in RNAi design will help improve the potency and selectivity of siRNAs, reduce toxicity and off-target effects, and will help bring RNAi to the clinic.

Finally, to overcome the challenges posed by transient and low level of gene silencing, multipronged efforts are underway to not only improve delivery by designing novel gene carriers, but also significantly modify siRNA backbone and make improvements in shRNA expression cassettes. Improved understanding of structure-function relationship of siRNA sequences will also lead to better design. Chemical modifications of siRNA molecules can also reduce both specific and non-specific off-target effects. We predict that successful elucidation of the detailed mechanism of RNAi coupled with better design and delivery systems will increase the range of applications for RNAi-based treatments, including effective clinical therapies.

ACKNOWLEDGMENTS & DISCLOSURES

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