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Section 2

GENE REGULATION

6

In Vivo Application of RNA Interference: From Functional Genomics to Therapeutics

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ABSTRACT

RNAi has rapidly become a powerful tool for drug target discovery and validation in cell culture, and now has largely displaced efforts with antisense and ribozymes. Consequently, interest is rapidly growing for extension of its application to *in vivo* systems, such as animal disease models and human therapeutics. Studies on RNAi have resulted in two basic methods for its use for gene selective inhibition: 1) cytoplasmic delivery of short dsRNA oligonucleotides (siRNA), which mimics an active intermediate of an endogenous RNAi mechanism and 2) nuclear delivery of gene expression cassettes that express a short hairpin RNA (shRNA), which mimics the micro interfering RNA (miRNA) active

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intermediate of a different endogenous RNAi mechanism. Non-viral gene delivery systems are a diverse collection of technologies that are applicable to both of these forms of RNAi. Importantly, unlike antisense and ribozyme systems, a remarkable trait of siRNA is a lack of dependence on chemical modifications blocking enzymatic degradation, although chemical protection methods developed for the earlier systems are being incorporated into siRNA and are generally compatible with non-viral delivery systems. The use of siRNA is emerging more rapidly than for shRNA, in part due to the increased effort required to construct shRNA expression systems before selection of active sequences and verification of biological activity are obtained. In contrast, screens of many siRNA sequences can be accomplished rapidly using synthetic oligos. It is not surprising that the use of siRNA *in vivo* is also emerging first. Initial *in vivo* studies have been reported for both viral and non-viral delivery but viral delivery is limited to shRNA. This review describes the emerging *in vivo* application of non-viral delivery systems for RNAi for functional genomics, which will provide a foundation for further development of RNAi therapeutics. Of interest is the rapid adaptation of ligand-targeted plasmid-based nanoparticles for RNAi agents. These systems are growing in capabilities and beginning to pose a serious rival to viral vector based gene delivery. The activity of siRNA in the cytoplasm may lower the hurdle and thereby accelerate the successful development of therapeutics based on targeted non-viral delivery systems.

I. INTRODUCTION

A. RNA interference (RNAi): strong and selective gene inhibition

RNA interference has rapidly displaced antisense and ribozymes as the preferred means for sequence-specific gene inhibition in cell culture studies (Bantounas *et al.*, 2004; Lu *et al.*, 2003; McManus and Sharp, 2002). Along with rapid adoption as a tool for functional genomics, expanding studies on RNAi itself have greatly enhanced our understanding of this endogenous gene inhibition process since discovery that posttranscriptional gene silencing (PTGS) in plants is also active in animal cells. Despite rapidly expanding studies, RNAi biology is still far from being well understood. A major challenge is the growing appreciation that the RNAi system is involved in several endogenous activities with differing roles.

One of the earliest recognized roles of RNAi is as an antiviral response triggered by the double-stranded (ds) RNA genome of the double-stranded RNA virus. For such a response to be effective, strong and selective gene

inhibition is important. This gene inhibition function operates through an active intermediate that is short fragments of the dsRNA genome, now called short interfering RNA (siRNA), as shown in Fig. 6.1. The RNAi antiviral response generally is accompanied by interferon and other events induced by recognition of the dsRNA viral genome. An important finding has been that introduction of siRNA with sequences matching endogenous genes, instead of invading virus genomes, leads to activation of the RNAi system and inhibition of that endogenous gene. Importantly, studies have shown that introduction of these artificial siRNA usually avoids the interferon and other concomitant biological responses (McManus and Sharp, 2002). Also, the RNAi machinery has been found to perform its function in the cytoplasmic compartment, reducing the hurdles for intracellular siRNA delivery compared to gene therapy requirements for delivery to the nucleus.

Another major RNAi role, but one that remains poorly understood, is the regulation of cellular activity by modulating endogenous gene expression. This function operates, at least in part, through an active intermediate that is a short expressed RNA with an imperfect palindrome sequence, now called

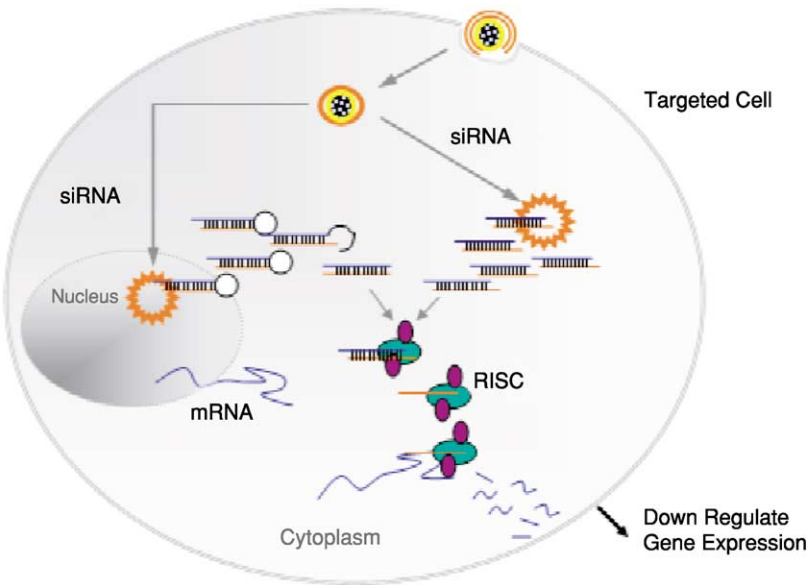

Figure 6.1. The two major pathways of RNAi can be invoked by delivering synthetic siRNA duplexes to the cytoplasm or delivering expression cassettes to the nucleus that produce "short hairpin" shRNA exported to the cytoplasm, either of which are taken up by cytoplasmic RISC machinery to down-regulate expression of the targeted gene.

microinterfering RNA (miRNA). The palindrome sequence forms an imperfect dsRNA segment with a loop of single-stranded (ss) RNA at one end, or a hairpin type of structure, mimicked by expressed short hairpin RNA (shRNA). This RNAi role regulating endogenous gene expression may help explain why the introduction of siRNA matching endogenous genes shows strong and selective inhibition of matching endogenous genes.

Thus while the specific role of RNAi can vary considerably, the fundamental activity of RNAi is blocking expression of specific genes. Consequently, it is not surprising that different RNAi processes have very similar mechanisms of action and share machinery. The shared machinery may aid efforts to harness RNAi for our own purposes by allowing greater utility of the techniques developed. However, with versatility comes complexity and increased risk of inducing unwanted or unexpected RNAi activities. Ultimately, though, what makes the RNAi system so attractive is its fundamental capabilities to achieve very strong inhibition of specific genes, and with striking selectivity. This is clearly essential for biological systems to regulate themselves. It is also the goal of targeted therapeutic strategies, driven by realization that the most successful drugs act through inhibition of specific target proteins. A number of studies have evaluated the extent of gene inhibition specificity achieved with siRNA, in most cases indicating selectivity but not an absolute selectivity (Chi *et al.*, 2003; Jackson *et al.*, 2003; Kariko *et al.*, 2004; Semizarov *et al.*, 2003; Sledz *et al.*, 2003). In fact, it can be difficult to distinguish between an off-target effect on the wrong gene and downstream biological consequences from RNAi silenced genes. An important aspect that has yet to be addressed is the relative selectivity by siRNA versus other gene sequence-based methods for inhibiting gene expression, i.e., antisense. Nonetheless, while off-target effects have been observed, the most common outcome is a finding of substantial siRNA gene selectivity. Thus the use of appropriate controls is important.

So far, the common feature found in RNAi processes is reliance on a short RNA oligonucleotide whose sequence determines the gene to be inhibited. This feature has fueled a rapid adoption of RNAi for biology research through the design of siRNA with a sequence matching genes of interest. The siRNA oligonucleotides were originally identified as short dsRNA fragments generated by RNAi machinery called Dicer that processes long dsRNA, such as from a viral infection, into siRNA fragments. These siRNA were found to be an active intermediate used a second piece of the machinery, called RISC, to select mRNA for degradation, as described elsewhere (Bantounas *et al.*, 2004; Lu *et al.*, 2003; McManus and Sharp, 2002). The mechanism for miRNA appears to be similar but not identical to that of siRNA. One similarity is that the miRNA also appear to invoke some of the same RISC components and degrade the homologous mRNA before ribosomal translation can occur. The endogenous activity of RNAi to regulate endogenous gene expression, which clearly depends on both good

specificity and strong inhibition, may explain its robust potency and selectivity. But while the nature of RNAi remains an area of active investigation, the use of short dsRNA sequences is proving to be an extremely robust and effective method to inhibit specific genes of interest, simply, and depending largely on the identification of unique sequences for that gene or splice variant (Fig. 6.1).

B. RNAi applications

In a short period of time, the use of siRNA to down-regulate expression of a specific gene has become the method of choice for cell culture or *in vitro* studies. Delivery of the siRNA duplex specifically targeting certain genes of interest in the cell tissue typically has been performed with siRNA targeting an individual gene, but multiples of siRNA duplexes inhibiting groups of genes are also possible. Beneficial or detrimental effects of siRNA inhibition of specific genes and inducing phenotypic changes in cells are analyzed using various means, including biochemical, pharmacological, and histological assays.

Currently, the use of siRNA to characterize gene function, and in particular exploration for potential therapeutic drug targets, is spreading over every aspect of biological research (Fig. 6.2). This phenomenon results from two basic realities: (1) siRNA is proving to be a very potent, robust, and easy to use inhibitor and (2) down-regulation of individual genes is a powerful tool for understanding the biological function of genes and biochemical pathways in the

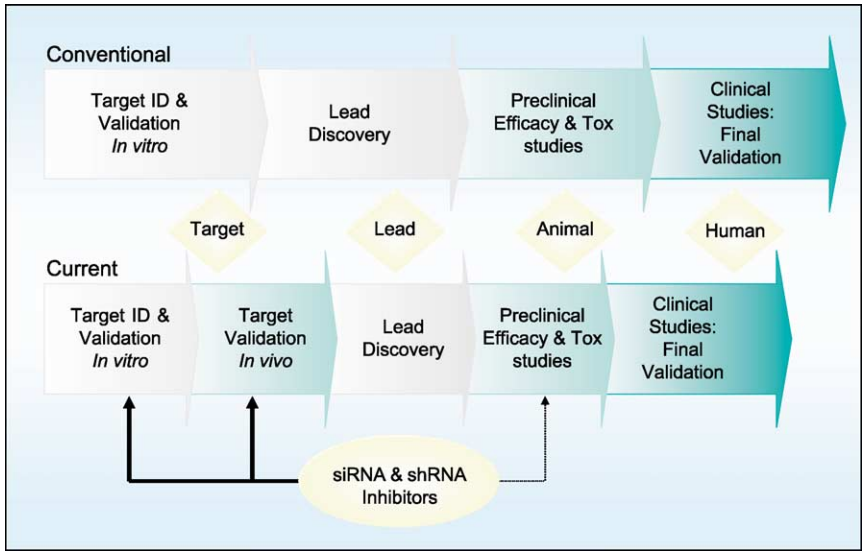

Figure 6.2. RNAi in Drug Discovery and Therapeutic Development.

development and control of pathology. The latter has led to considerable adoption of siRNA for functional genomics looking for inhibition of genes that can generate therapeutic benefits reversing pathological effects, often caused by overexpression of those same genes. However, the approach clearly depends on the effectiveness of siRNA delivery to modulate, very selectively, the expression of specific genes and, as a result, to induce physiological or pharmacological effects. A key requirement is identification of an effective site in the target mRNA sequence for siRNA accessibility. Also, for most applications, effective transfection of the siRNA agent into the cytoplasmic compartment is critical (Bantounas *et al.*, 2004; McManus and Sharp, 2002).

Not surprisingly, cancer research is a dynamic and exciting area for the application of siRNA inhibitors (Lu *et al.*, 2003). However, research in many different therapeutic areas is invoking application of siRNA for use as a research tool for the validation of gene functions (Xie *et al.*, 2004). Regardless of therapeutic area, the success of *in vitro* transfection of siRNA into cells for target research has led to a strong interest in extending those studies to *in vivo* systems. However, *in vivo* delivery of siRNA into specific tissues of animal disease is much more complicated. Although increasing numbers of studies on target identification and validation using siRNA *in vitro* have been reported, limited reports of *in vivo* studies have indicated a lack of effective delivery methods for siRNA agents. The key to *in vivo* application is a delivery system that transports the siRNA into the target tissue and into the cell cytoplasm, or shRNA expression cassette to the nucleus much like the dependence of gene therapy on appropriate delivery methods. Given the extensive efforts in nucleic acid delivery for gene therapy, many delivery tools can be considered for siRNA or shRNA delivery, including biological-based vectors and synthetic chemical- and physical-based systems.

A consequence of the fast-growing literature on using siRNA as a research tool for functional genomics is an emerging interest in siRNA as a therapeutic. Therapeutic applications, even more than functional genomics, clearly depend on optimized local and systemic delivery of siRNA *in vivo*. Therefore, means for delivering siRNA into targeted tissues so as to maintain its activity within targeted cells and on the targeted gene sequence are the key aspects considered here in order to fulfill the goals of both functional genomic research and therapeutic development.

II. DELIVERING SHORT INTERFERING RNA (siRNA) *IN VIVO*

The effectiveness of siRNA *in vitro* has been demonstrated by inhibiting expression of many different genes and in many different cell types. In virtually all cases, the results depend on means for intracellular delivery, although

transfection of siRNA into cultured cells is relatively easy compared with plasmids and other gene expression systems (Bantounas *et al.*, 2004; McManus and Sharp, 2002). A wide variety of nucleic acid delivery systems have been developed, including viral vectors and "non-viral" approaches, achieving efficient and significant modulation of gene expression for many types of cells. Regardless of whether the systems are biologics such as shRNA expression vectors (Davidson and Paulson, 2004; Pardridge, 2004) or chemical agents delivering synthetic siRNA, the RNAi tools can be used to decrease gene expression and, if the gene is important, result in phenotypic changes. The extension of *in vitro* success to *in vivo* systems is emerging, but further improvement remains a critical need for application of siRNA to drug target research, and potential clinical application.

A. "Non-viral" RNAi agent classes

Several methods have been developed to utilize the endogenous RNAi machinery to knock down specific genes of interest. So far, all the available methods rely on introducing, by one means or another, a short double-stranded RNA oligonucleotide matching a part of the gene of interest. The original method relied on introduction into cells of a synthetic dsRNA oligonucleotide that resembles the natural siRNA oligonucleotides. The artificial siRNA is taken up by the RISC machinery and used to block the expression of endogenous mRNA containing a matching sequence. This approach remains the most prominent method for high-throughput RNAi research, as short oligonucleotide synthesis can be fast and performed in high throughput. It is limited primarily by the cost of synthetic oligonucleotides and the need for repeated chemical synthesis.

A growing effort has developed around methods to deliver an expression cassette to produce the RNAi intermediate. An initial challenge for this approach was the difficulty of getting expression of two short complementary RNA oligonucleotides within the cell to result in their hybridization to yield the dsRNA oligonucleotide. One solution to this problem is expressing a single RNA with a palindromic sequence so that it can hybridize with itself and form a stem–loop "hairpin" structure much like miRNA, now called short hairpin RNA (shRNA). Theoretically, such expression cassettes can be introduced by any of the standard gene delivery systems, including viral and non-viral methods. A growing number of efforts are being reported for the construction of such systems and evaluation for *in vivo* application (Davidson and Paulson, 2004; Devroe and Silver, 2002; Kobayashi *et al.*, 2004; Pardridge, 2004; Tiscornia *et al.*, 2003; Xia *et al.*, 2002) However, the time and effort needed to construct such expression cassettes and incorporate them into delivery systems add considerably to the time and cost to set up a study for a gene of interest. This is particularly true when it is realized that several sequences must be tested to find

an effective sequence. Unfortunately, identification of active sequences using synthetic siRNA oligonucleotides is not always predictive of active shRNA sequences, reducing the utility of preliminary siRNA screens to address this problem. Once an effective expression cassette is identified, the materials usually can be replenished through standard molecular biology techniques.

B. "Non-viral" delivery systems

A wide range of nucleic acid delivery systems have been devised without adaptation of virus and thus fall into the broad category called "non-viral" vectors. Lumped together in this poorly defined class are many different systems covering vastly different forms of nucleic acid. Likewise, this class covers a wide variety of methods to formulate and deliver the nucleic acid to enable intracellular delivery, including cationic complexes with lipids, polymers, or both, PLGA microsphere depot formulations, hydrophilic protective polymers, physical force-based delivery such as bombardment with nucleic acid-coated gold particles or electroporation, and many other delivery methods. Note that a common feature conveyed by the non-viral category is their operability with either natural or synthetic forms of the nucleic acid, e.g., from plasmids to phosphorothioate antisense oligonucleotides. In addition, the success of each for delivery *in vivo* depends on many factors, such as route and tissue uptake. The greatest success has been attained for local delivery to tissues, e.g., PLGA microspheres and electroporation for muscle delivery. Only a few successes have been attained for systemic delivery such as high-pressure tail vein administration or "hydrodynamic" delivery for liver hepatocytes and the DOTAP lipoplex for lung tissues. Some of these delivery methods have limited potential application in humans, e.g., hydrodynamic delivery, but their capabilities can be enabling for functional genomics research investigations *in vivo*, where the criteria for success are very different (Lu *et al.*, 2003).

One of the important non-viral delivery methods is based on the use of cationic transfection agents to form complexes with oligonucleotides, and these methods have been shown to be active with siRNA *in vivo*. The most commonly used transfection reagents have been cationic lipids, producing lipoplex complexes (Spagnou *et al.*, 2004). Sorensen *et al.* (2003) examined delivery with cationic liposome-based administration in mice of siRNAs combined with a matching plasmid encoding the green fluorescent protein (GFP) and found inhibition *in vivo* of the GFP plasmid gene expression. Furthermore, the studies evaluated the intraperitoneal injection of anti-TNF-α siRNA inhibited lipopolysaccharide-induced tumor necrosis factor-α (TNF-α) gene expression, whereas secretion of interleukin (IL)1-α was not inhibited. As found in gene therapy efforts, development of non-viral delivery methods that achieve tumor-targeted siRNA delivery and activity from an intravenous administration has proven

to be difficult. One study reported use of a cationic chemical derivative of cardiolipin to form lipoplexes with siRNA targeting the cRAF (also known as RAF-1) oncogene that inhibited tumor growth in a sequence-specific manner but the study neglected to evaluate whether the target gene was specifically inhibited (Chien *et al.*, 2004). This confirmation of target inhibition can help reveal effects that cannot be attributed to an RNAi mechanism as found in earlier studies using an aqueous administration of siRNA (Filleur *et al.*, 2003).

The cationic lipoplex and polyplex systems have formed the foundation for numerous efforts to develop ligand-targeted gene delivery systems. Studies have begun to emerge for adaptation of these systems for siRNA (Kakizawa *et al.*, 2004; Khan *et al.*, 2004; Shiffeleres *et al.*, 2004b). Results obtained using ligand targeting of plasmids to tumor neovasculature suggest that systemic tumor therapeutics using gene delivery is a possibility (Hood *et al.*, 2002; Ogris *et al.*, 2003; Walker, 2005; Woodle *et al.*, 2001), but this approach focuses on delivery to endothelial cells of the tumor blood vessels. The efficiency of plasmid expression is also limited, as trafficking to the nucleus is thought to be inadequate. For siRNA, its activity in the cytoplasm reduces the challenge. Results have been published using systemic administration of a targeted nanoplex using an Arg-Gly-Asp (RGD) motif peptide ligand that combines selective localization at neovasculature with delivery of siRNA inhibiting the vascular endothelial growth factor (VEGF) pathway gene expression driving angiogenesis whether in tumors or eye disease (Kim *et al.*, 2004; Schiffelers *et al.*, 2004b). Importantly, in both studies, results showed siRNA sequence-specific target gene inhibition in the pathological tissue. Such systems introducing dual targeting offer the promise of increasing selectivity in targeted therapeutic development. These advances in cationic complexes forming layered nanoparticles appear promising for the therapeutic application of siRNA for metastatic cancer and many other angiogenesis-related diseases.

Non-viral vectors have many advantages, especially for therapeutics, over viral vectors. For example, immunogenicity of viral vectors has precluded multiple administrations and resulted in severe toxicity limitations. Chromosomal integration, and the resulting safety concerns, is also avoided by non-viral vectors. While potential safety issues are not a limitation for functional genomics, immunogenicity and other side effects can obscure the results and need to be avoided. Clearly, effective siRNA delivery is crucial for *in vivo* functional genomics, i.e., inhibition of gene expression in a significant number of cells as a means to emulate the biological effect of a drug targeting the same protein can be emulated. This means that the siRNA needs to be delivered to approximately the same percentage of the same cells and the cells in the same tissue as will be affected by the candidate drug. As mentioned for viral vectors, an equally important requirement for non-viral vectors is delivery without significant

background activity from the delivery method itself. Non-viral delivery methods tend to have much lower levels of biological effects, other than that from the gene, relative to viral vectors with their many protein components.

C. *In vivo* siRNA delivery

Delivering RNAi agents *in vivo* basically falls into two approaches: local and systemic administration. Each of these approaches can be uniquely suitable for a particular tissue type or biological study system. Or, they can be used independently to reach the same targeted organ as a means to verify that the observed gene inhibition outcome is not due to the delivery method. For example, skin and muscle can be better accessed using local delivery, whereas lung and tumor can be reached efficiently by both local and systemic deliveries. The choice between local and systemic delivery largely depends on what tissues and cell types are targeted and the expected outcome for siRNA-mediated gene knockdown in terms of biological readout. In addition, the choice usually is only a part of the entire consideration of study design involving vector carriers, administration routes, and approaches for siRNA delivery *in vivo*. Increasing data show that siRNA is a very potent sequence-specific inhibitor in many tissue types.

1. Local administration

a. Ocular

An increasing number of clinical protocols are appearing for treating eye diseases with nucleic acid drugs such as antisense or RNA aptamers. These clinical delivery approaches appear suitable for siRNA administration but depend on local administration. Using a model of retinal neovascularization induced by laser damage, Reich and colleagues (2003) delivered nonformulated siRNA specific to murine VEGF to the subretinal space and observed significant reduction of eye angiogenesis. Importantly, unlike antisense or RNA aptamers, this work indicated that chemical protection of the siRNA was not essential, at least in the intravitreal compartment of the eye. Using a different murine model, one for herpetic stromal ketatitis, which develops from herpes simplex virus infection, the laboratory of Barry Rouse also reported the use of polyplexes for local administration of siRNA to inhibit the VEGF pathway (Kim *et al.*, 2004). This work compared local with systemic targeted delivery, as discussed later. It now seems clear that local ocular delivery of siRNA, through different routes with different formulations, can be used to inhibit genes sufficiently for gene function studies. The work also provides strong support for the therapeutic application of siRNA for ocular disease, especially ocular neovascularization.

b. Brain

The brain tissues are the foundation of the central nervous system (CNS), obviously a very important biological system and one representing considerable interest for both functional genomics and therapeutics. This tissue is one in which capabilities to inhibit genes selectively *in vivo*, for either application, are especially critical, as nerve function depends on the actual nerve network within the brain (Buckingham *et al.*, 2004). Initial efforts for brain delivery focused on expressed RNAi constructs (Davidson and Paulson, 2004), but Dorn *et al.* (2004) evaluated the infusion of aqueous solutions of chemically protected siRNA oligonucleotides directly into the brain and found that selective gene inhibition could be obtained. However, the special nature of the latter method, including specialized chemical modifications of the siRNA and surgically implanted infusion pumps delivering high doses, limits its usefulness for many laboratories interested in this area. Other studies on the use of cationic formulations for the delivery of siRNA to brain found that delivery was more effective with the tested lipoplex than with the tested polyplex (Hassani *et al.*, 2004). The success observed in these diverse studies shows the promise of siRNA delivery to brain tissues for functional genomics and, in the long term, good potential for therapeutic applications.

c. Tumoral

For functional validation of the tumorigenic genes, intratumoral delivery of siRNA is a very attractive approach. The rapid extension of siRNA functional genomics studies *in vitro*, now very well accepted, to studies in preclinical human xenograft tumor models (Ding *et al.*, 2004), also well accepted as a key stepping stone to clinical investigation, is limited only by a lack of good local tumor delivery. Unfortunately, like earlier gene therapy studies, local delivery of siRNA into tumors is not efficient with either aqueous solutions (so-called "naked" nucleic acid) or standard transfection reagents, including the best cationic lipids and polymers. In fact, local administration of aqueous siRNA into tumors was found less effective than a distal systemic administration on an endogenous reporter gene (Filleur *et al.*, 2003). In that study, effects observed from distal administration were not attributable to an RNAi activity and the authors concluded that tumor delivery was inadequate. Some work has begun to emerge for the local tumor delivery of siRNA, showing sufficient gene inhibition for target validation (Lu *et al.*, 2002; Minakuchi *et al.*, 2004).

d. Pulmonary

Non-viral delivery of nucleic acid into the airway has been a very active area, in part due to intense efforts for the development of therapy to treat cystic fibrosis patients. Because of an overwhelming immune response to adenoviral vectors,

efforts for pulmonary nucleic acid delivery turned to non-viral carriers to draw upon their generally reduced toxicity and immune response. Unfortunately, non-viral methods have not yielded positive results in clinical investigations, but may be more effective with siRNA given its lack of dependence on nuclear delivery.

Evidence has appeared that siRNA can effectively knockdown endogenous genes (Zhang *et al.*, 2003) and viral proteins of a group of RNA viruses, e.g., influenza (Ge *et al.*, 2003; Tompkins *et al.*, 2004) and SARS coronavirus (Li *et al.*, 2005), resulting in significant effects of antiviral infection in various mammalian cell systems. The *in vitro* proven siRNA for influenza were evaluated for *in vivo* pulmonary delivery using standard non-viral vectors and found to inhibit influenza virus pulmonary infection (Ge *et al.*, 2004; Tompkins *et al.*, 2004). However, these delivery methods are not strong candidates for clinical application and better methods are needed. In a separate study with a clinically feasible intranasal delivery system and using SARS virus siRNA, results using a primate disease model found good evidence of both safety and efficacy (Li *et al.*, 2005). Thus it appears that the many non-viral gene delivery systems are applicable for pulmonary delivery of siRNA.

e. Skeletal–muscular

The tissues of the skeletal–muscle system, the joints and skeletal muscles, are relatively accessible for local administration methods. Thus the key to using siRNA sequences directed to any gene target implicated in rheumatic and musculoskeletal diseases is effective local delivery enabling intracellular delivery of siRNA agents at the diseased site and, more specifically, the specific cellular components of the disease pathology (Rutz and Scheffold, 2004). Direct injection of siRNA molecules has been reported by hydrodynamic pressure in isolated limbs (Hagstrom *et al.*, 2004) or formulated with cationic lipids or polymers can be considered for local delivery, but tend to induce inflammation, which is contraindicated for arthritic disease studies. Also, for plasmid delivery to skeletal muscle, eletroporation has proven the most robust method and recently applied to siRNA delivery, (Golzio *et al.*, 2004). The extension of this method to inflamed joints in an arthritis model has shown promise as a method for functional genomic studies (Schiffelers *et al.*, 2005), but clinical application of this approach has yet to be described. Ultimately, the biology of the inflammation and immune response, which is central to the rheumatic arthritic diseases, may benefit from cell targeting. To that end, the use of ligand-targeted nanoparticles, even with local intraarticular injection, is attractive as a potential means to achieve gene inhibition without exacerbating the pathological phenotype by the delivery system itself.

2. Systemic administration

a. Liver targeting

Some of the first published results showing activity of siRNA in mammals accomplished that feat by delivery into mouse liver. This was achieved using hydrodynamic delivery, a rapid injection of a large volume of aqueous solution into the mouse tail vein creating high pressure in the vascular circulation that leads to extensive delivery into hepatocytes (Lewis *et al.*, 2002; McCaffrey *et al.*, 2002; Song *et al.*, 2003; Zender *et al.*, 2003). Studies using hydrodynamic administration also found poor activity of aqueous siRNA when given by traditional intravenous administration, even when chemically stabilized siRNA were used (Layzer *et al.*, 2004), unlike earlier findings with antisense oligonucleotides. These studies showed that siRNA not only can inhibit exogenous plasmid expression, but also endogenous hepatocyte gene expression. The success implies that delivery of siRNA to the liver results in uptake by a majority of hepatocytes, as found for plasmid delivery. Interestingly, recent studies found that aqueous administration of siRNA didn't induce a non-specific interferon response (Heidel *et al.*, 2004). The hydrodynamic administration procedure allows the use of siRNA in mice for gene function and drug target validation studies (Sen *et al.*, 2003; Zender *et al.*, 2003), but is limited largely to research on liver function and metabolism or liver infectious diseases such as hepatitis. Although this particular method is not clinically feasible for human patients, it is an effective approach for gene function validation. Song *et al.* (2003) injected Fas siRNA and achieved down-regulation of both Fas mRNA and Fas protein in mouse hepatocytes, and the effects persisted without diminution for 10 days. Zender and colleagues (2003) delivered 21 nucleotide siRNAs against caspase 8, resulting in inhibition of caspase 8 gene expression in the liver, thereby preventing Fas (CD95)-mediated apoptosis. Giladi and colleagues (2004) used hydrodynamic delivery of siRNA to inhibit levels of hepatitis B viral transcripts, viral antigens, and viral DNA in liver and sera. Adenovirus has also been applied for liver delivery of expressed RNAi methods but mainly offers an orthogonal delivery approach to confirm that findings are due to gene inhibition and not specific to the delivery method. Although both hydrodynamic delivery and adenoviral vector are not clinically acceptable methods, these two systems have a relatively high hepatocyte efficiency, which is a very powerful tool for hepatocyte functional genomic studies.

Targeted systems for siRNA delivery into liver have also been a very attractive approach and are under development (Ren *et al.*, 2001). Clearly, such liver-targeted delivery systems are more clinically feasible for the development of siRNA-based therapeutics for the treatment of various liver-related metabolic

and hepatitis viral diseases. One alternative method for liver-targeted delivery of siRNA is to use chemical modification of the oligonucleotide, as suggested by work with cholesterol conjugates (Soutschek *et al.*, 2004). However, this work shows that at least three challenges must be addressed: adequate protection of the siRNA oligonucleotide from biological degradation en route to the liver, protection of the siRNA oligonucleotide from rapid glomerfiltration by the kidney into the urine (actually this is more important than stabilization), and selective uptake by the target hepatocytes. The use of cholesterol conjugates was found to require very high doses, suggesting that the distribution was widespread rather than liver targeted. Further efforts to attain liver targeted siRNA are warranted.

b. Tumor and neovasculature targeting

Although primary tumors often can be reached by local administration, malignant tumors grow fast and spread throughout the body via blood or the lymphatic system. Their unpredictable and uncontrolled growth makes malignant cancer the most dangerous and fatal form of cancer. Metastatic tumors established at distant locations usually are not encapsulated and thus are more amenable for systemic delivery. Therefore, local delivery of siRNA or any therapeutic agent is limited to a few tumor types, such as head-and-neck cancer and melanoma. For functional genomics, local administration methods (as discussed earlier) can meet the requirements for most studies by acting on primary tumors, even in xenograft models, which form the basis of most cancer biology research. However, for therapeutics, systemic delivery of siRNA is needed.

Systemic delivery imposes several requirements and greater hurdles than local delivery. For siRNA, it must provide increased oligonucleotide stability in the blood and in the local environment before it enters the target cells. In addition, it often requires the siRNA to pass through multiple tissue barriers to reach the target cell and may benefit from protection from loss of activity once within the target cells. Sorenson *et al.* (2003) described the use of lipoplex gene delivery systems for the systemic delivery of siRNA, attaining a strong lung delivery as found when plasmid DNA is used. Other work with siRNA lipoplexes formed with a cardiolipid analogue showed phenotypic effects on tumor growth but lack confirmation of an RNAi activity at the tumor and suggest that much of the distribution is to the lung as expected from a lipoplex. An emergence of targeted nanoparticle systems, initially for plasmids or DNA oligonucleotides for cancer, is much more promising (Hood *et al.*, 2002; Pun *et al.*, 2004; Woodle *et al.*, 2001). The nature of these systems to protect and deliver isolated DNA suggests that they can be adapted for siRNA oligonucleotides. Results support this conclusion using an RGD peptide ligand-directed nanoparticle for antiangiogenic treatment for cancer (Dubey *et al.*, 2004) by

systemic siRNA delivery (Schiffelers *et al.*, 2004b). These results provide growing support for the development of systemic siRNA treatments for cancer, with a growing proof of concept for antiangiogenic modalities as reviewed elsewhere (Lu *et al.*, 2005). The RGD ligand-targeted nanoparticle for targeting neovasculature has also been studied in ocular neovascularization models (Kim *et al.*, 2004). The results further strengthen the support for this approach as a clinically feasible method for siRNA therapeutics. They also provided a demonstration that siRNA for several genes can be combined in the same nanoparticle to give a better inhibition of disease pathology.

Of course a direct means to translate systemic gene therapy delivery methods to RNAi is through the use of shRNA expression methods. At this time though the advantages and disadvantages of systemic tumor delivery for expression-based RNAi versus siRNA oligonucleotide-based RNAi have yet to be fully elucidated. One clear advantage of siRNA, however, is rapidity with which different siRNA sequences and the matching genes can be studied. Importantly, the siRNA facilitates rapid studies of the interactions of genes, as combination of siRNA oligonucleotides is easy and effective (Kim *et al.*, 2004).

III. *IN VIVO* siRNA APPLICATIONS

A. *In vivo* functional genomics

The traditional approach to identifying genes involved in specific biological processes begins with the determination of genes correlating with the occurrence of that process, i.e., genes that are up- or down-regulated in cells and tissues of the biological system of interest. When applied to pathological processes, this approach generates large pools of up-regulated genes that are candidates as drug targets. However, the success of this approach is limited, as correlation is insufficient for causation or for efficacy. Consequently, a tool that can selectively down-regulate individual genes within the cells and tissues of a pathological process is widely recognized as a valuable means to understand gene function, especially for facilitating the discovery of protein targets for drug intervention. Because siRNA is proving to be a potent and robust means for this important objective, it is being rapidly adopted as the preferred functional genomics tool, at least in cell culture (Buckingham *et al.*, 2004).

One of the key hurdles for drug target discovery or validation directly in animal disease models has been a lack of effective *in vivo* nucleic acid delivery methods. While antisense activity in animals has been limited in general, intravenous administration of aqueous formulations has been used to inhibit liver gene function. Using hydrodynamic delivery of siRNA (Layzer *et al.*, 2004; Lewis *et al.*, 2002; McCaffrey *et al.*, 2002; Sen *et al.*, 2003) is one way to use

siRNA gene inhibition for liver gene function. A key element of the challenge is often a large amplitude of inhibition (or overexpression) of the gene for a significant effect on phenotype to be observed. Requirements for large phenotypic effects appear to be a bigger challenge for therapeutics than for gene function studies, particularly for several classes of genes and proteins.

In cancer, the tumorigenesis process is thought to be the result of an abnormal overexpression of oncogenes, growth factors, and mutant tumor suppressors, even though underexpression of other proteins also plays critical roles. Efforts to identify and validate tumorigenic targets have been focused mainly on those targets overexpressed in the tumor tissues and promoting tumorigenesis as a means to enable development of small molecule and antibody anticancer drugs acting through an inhibitor mechanism. There are rapidly increasing numbers of reports demonstrating that siRNA and shRNA are able to knockdown tumorigenic genes *in vitro*, with emerging reports for *in vivo* (Lu *et al.*, 2003b; Xie *et al.*, 2004). Studies designed to reveal whether a gene target plays a tumorigenic role use siRNA duplexes specifically targeting its mRNA sequence to knock down its expression and then observing whether the effect on pathology is a direct and specific effect. One method has been described for a unique target identification approach (Lu *et al.*, 2003a) named Efficacy-First discovery. This method utilizes efficient nucleic acid delivery into xenograft tumors to induce phenotypic effects on tumor growth rate as a method to verify or validate candidate genes for a controlling role in tumor growth behaviors. Using these methods, a pool of gene targets was identified for changes in expression that correlated with changes in tumor growth rate, i.e., their expression correlated with efficacy. Among the well-known cancer cell growth factors, VEGF and VEGF R2 represent two of the most widely recognized and highly validated targets. In these studies, siRNA-mediated downregulation of these endogenous genes in clinically relevant animal models was used as part of a functional genomics study in cancer (Lu *et al.*, 2003a). Marked tumor growth inhibition was observed following repeated delivery of the siRNAs specific to hVEGF and mVEGFR2, which accompanied knockdown of the growth factor at both mRNA and protein levels. The results illustrate the feasibility of using siRNA delivery in animal tumor models for drug target validation according to its ability to achieve efficacy (Lu *et al.*, 2003a). This *in vivo* delivery of siRNA enables differentiation of genes to find out which play a disease-controlling role in tumor growth. This validation process, called disease-control validation, is based on tumor efficacy by siRNA *in vivo* and with clinically relevant xenograft tumor models to give a clear indication of the importance of specific proteins as a drug target (Lu *et al.*, 2003a).

Once siRNA studies in cell culture provide a better understanding of the mechanism of action of candidate gene targets, they lay the foundation for use of the siRNA in a disease model. To be effective, *in vivo* functional genomics requires

high efficiency *in vivo* delivery and good activity in a clinically relevant disease model for the clinical indication of interest. Results obtained to date indicate that the gene inhibition of siRNA is not only a sequence-specific effect, but is also sustainable and obtainable with relatively few side effects (low noise level). Importantly, this approach offers an ability to gain insight into the genes and proteins associated with the later stages of pathology, as it can be applied to established disease tissues. Such capabilities to move rapidly from cell culture into clinically relevant disease models promise to revolutionize the speed with which drug targets can be identified and validated for drug discovery. Equally important, the use of siRNA *in vivo* offers the prospects for rapidly obtaining a better understanding of how a particular target can be used to achieve a clinically meaningful therapeutic intervention in disease. This capability is also important for basic research into disease biology, since selective gene inhibition tools and ones that can be constructed quickly and that can operate in animal models will facilitate the rapid expansion of our knowledge. Thus the emerging capability of *in vivo* delivery of siRNA is greatly expanding its power as a tool for functional genomics.

B. Therapeutics

The specificity and potency of siRNA in cell culture and in animal studies suggest that it may be useful as a therapeutic agent (Schiffelers *et al.*, 2004a). However, the development of siRNA as a therapeutic agent faces a number of challenges, especially for systemic routes of administration. The most critical hurdle for *in vivo* delivery is attaining adequate delivery to disease tissue and cells. A growing number of studies are being reported that evaluate the prospects of siRNA for therapeutics, as summarized in Table 6.1.

Surprisingly, *in vivo* stability has not proven to be the major rate-limiting barrier, even for intravenous administration. In fact, rapid excretion of siRNA from blood into the urine occurs before degradation. Nonetheless, efforts are underway to increase its biological stability, primarily via medicinal chemistry originally developed for oligonucleotides. However, chemical stabilization does not address key requirements for better pharmacokinetics and tissue distribution. With conjugation of lipophilic residues to increase serum protein binding, improved pharmacokinetics of the oligonucleotide, alteration of its biodistribution, and reduced urinary excretion were observed (Soutschek *et al.*, 2004). Ultimately, such chemical modification must also address requirements for entering targeted cells, overcoming endosomal and other intracellular barriers, and retaining activity with the cellular RNAi machinery. A different approach to solving these problems using ligand-targeted nanoparticles (Kim *et al.*, 2004; Pardridge, 2004; Schiffelers *et al.*, 2004b) is showing good activity even without chemical stabilization. The fundamental thinking behind this approach is that RNA interference is a natural process involving a complicated

Table 6.1. *In Vivo* Delivery of siRNA for Developing Novel Therapeutics

Therapeutic Applications	Target gene	Model	Delivery vehicle/route	RNAi phenotype and reference
Cancer therapy	VEGF	MCF-7/nude, MDA-MB-435/ nude	Polymer based, intratumoral injection	Reduction of VEGF and inhibition of tumor growth (Lu *et al.*, 2003a,b)
	VEGF R2	N2A/nude	Ligand-targeted nanoparticle, i.v. injection	Tumor growth inhibition (Schiffelers *et al.*, 2004)
	VEGF	PC-3/nude	Atelocollagen, intratumoral injection	Suppressed tumor angiogenesis and tumor growth (Takei *et al.*, 2004)
	VEGF	JT8/nude MDA-MB-231/ SCID	Naked siRNA, i.v., injection	Reduction in VEGF expression and inhibition of tumor growth (Filleur *et al.*, 2003)
	c-raf		Cationic cardiolipin analogue based liposome (CCLA), i.v., injection	Tumor growth inhibition (Chien *et al.*, 2004)
	RRM2	Orthotopic pancreatic/nude	Systemically administration	Suppressed tumor growth, increased tumor apoptosis and inhibition of metastasis through the synergism between RRM2 siRNA and gemcitabine (Duxbury *et al.*, 2004)
Ocular diseases	VEGF	Mice/laser photocoagulation	Saline, local injection	Inhibited choroidal neovascularization (Reich *et al.*, 2003)
	VEGF R1/R2	Mice/HSV induction	Ligand-targeted nanoparticle, i.v. injection	Anti-angiogenesis effect demonstrated by reduction of the neovasculature areas (Kim *et al.*, 2004)
	TGF-beta Rll	C57BL6 mice	Polymer-based, subconjunctival injection TransIT-TKO, local injection	Reduced the imflammatory response and matrix deposition, (Nakamura *et al.*, 2004)

Rheumatoid arthritis	TNF-α	Mice	i.a. local injection	Inhibition of collagen-induced arthritis (CIA) (Schiffeleres *et al.*, 2005)
Anti-viral therapy	Influenza A virus genes	C57BL/6 mice	PEI, i.v. administration	Reduced virus production in lungs of infected mice (Ge *et al.*, 2004)
	Influenza A virus genes	BALB/c mice	PBS, hydrodynamic i.v., injection; oligofectamine, intranasal administered.	Reduced lung virus titers in infected mice and protected animals from lethal challenge (Stephen *et al.*, 2004)
	SARS virus genes	Monkey	Intranasal administered	Inhibited SARS virus replication and reduced the SARS-like symptom in infected monkey (Li *et al.*, 2004)
	HBV genes	BALB/c mice	PBS, hydrodynamic i.v. injection	Inhibition in the levels of HBV viral transcripts, viral antigens, and viral DNA detected in the liver and sera (Giladi *et al.*, 2003)
CNS disease	P2X3	Rat models	Saline, intrathecal injection	Diminished P2X3 mRNA expression and P2X3 protein translocation, diminished pain responses, relieved chronic neuropathic pain (Dorn *et al.*, 2004; Ganju *et al.*, 2004)
	DAT	Mice	Saline, ventricular infusion	Down-regulation of DAT mRNA and protein in the brain. Elicited a temporal hyperlocomotor response (Thakker *et al.*, 2004)
	Alpha(2A)-ARs	Rat	Saline	Decreased the levels of both alpha (2A)-AR mRNA and [(3)H] RX821002 binding sites in the brainstem. Decreased anxiety in the adult animals (Shishkina *et al.*, 2004)

(*Continues*)

Table 6.1. *(Continued)*

Therapeutic Applications	Target gene	Model	Delivery vehicle/route	RNAi phenotype and reference
CNS disease (cont.)	GluR2, Cox-1	Rat	Electroporation	Reduction in the expression levels of both the mRNA and protein of target genes. Treated animals exhibits consistent physiological functions such as glutamate release from presynaptic sites, LTP and LTD (Akaneya *et al.*, 2005)
Others	TNF-α	Mouse	DOTAP, i.p. injection	Inhibited lipopolysaccharide-induced TNF-α expression and development of sepsis (Sorensen *et al.*, 2003; Sioud *et al.*, 2003)
	GAPDH	Mouse	InfaSurf, intranasal administration	Lowered GAPDH protein in lung, heart, and kidney by approximately 50–70 1 and 7 days after siRNA administration (Massaro *et al.*, 2004)
	HO-1	Mouse	Naked, intranasal administration	Enhanced apoptosis, via increased Fas expression and caspase 3 activity, in mouse lung during I-R injury (Zhang *et al.*, 2004)
	ApoB	C57BL/6 mice	Stabilized Chol-siRNA, i.v. injection	Silenced the apoB mRNA in liver and jejunum, decreased apoB protein levels in plasma, and reduced total cholesterol (Soutschek *et al.*, 2004)
	Caspase-8, caspase-3	C57BL/6 mice	10% lipiodol, high-volume portal vein injection	60% reduction in caspase-8 and caspase-3 expression, decrease ischemia/reperfusion injury to the liver (Contreras *et al.*, 2004)

cellular mechanism where siRNA is the predominant intermediate playing a sequence-specific silencing function. Therefore, preserving the biochemical authenticity of siRNA and improving its *in vivo* delivery efficiency with nanoparticle or other formulations will have the best chance for success in therapeutic application, and, importantly, the best chance to reduce unwanted side effects. Although local and topical delivery of siRNA may fit well for certain disease applications, the systemic delivery of siRNA will have much broader therapeutic applications. The ideal system for siRNA systemic delivery should be a nanoparticle with ligand-directed tissue localization (Fig. 6.3). This three-stage system should be able to first protect the siRNA duplex from excretion and degradation in body fluid such as blood and, at the same time, avoid aggregation and nonspecific binding of the nanoparticles. The system should also have targeting capability to reach disease tissue specifically. When the particle binds and enters the targeted cells, the siRNA content should be released for action. When the siRNA specifically inhibits expression of a disease-causing gene and protein, the clinical benefit will be achieved. As described earlier, a layered nanoplex system has been described that combines neovasculature-targeted

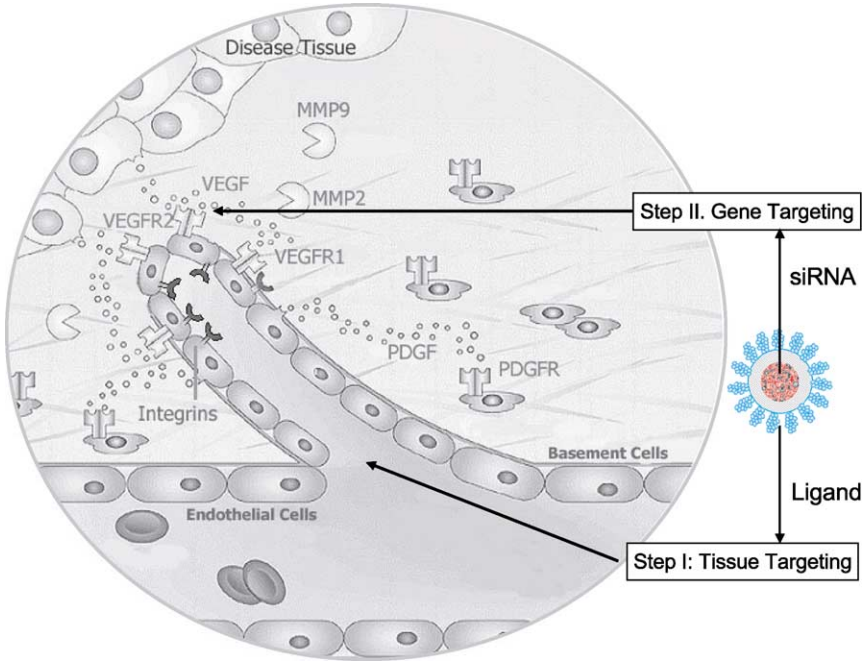

Figure 6.3. Dual-Targeted anti-angiogenesis siRNA systemically delivered using ligand-directed nanoparticle.

nanoparticles giving cytoplasmic delivery with highly potent siRNA active in the cytoplasm. This nanoparticle system appears promising for the therapeutic application of siRNA for metastatic cancer and many other angiogenesis related diseases. The modular design of this nanoparticle should permit incorporation of other ligands applicable for siRNA delivery for other diseases.

Using RNAi for therapy is attractive to directly silencing pathogenic genes or disease-causing mutant genes. As disease mechanisms become increasingly clear, its application can be developed to silence newly identified genes involved in known pathogenic pathways. For example, an obvious siRNA target for the treatment of Alzheimer's disease (AD) is the β-site APP-cleaving enzyme BACE, which is required for the production of Aβ peptide and is present at elevated levels in the cortex of people with AD.

One of the greatest prospects for therapeutics is the ability to develop therapeutic agents that control multiple targets. This is one aspect of siRNA that offers considerable promise relative to the current set of targeted therapeutics such as antibodies. Considerable evidence shows that many types of human diseases result from overexpression of multiple disease-causing genes. Thus the potent and specific properties of siRNA that are controlled solely by changes to their sequence suggest that their combinations will be easily managed. Kim *et al.* (2004) showed the facility with which siRNA can provide combination therapeutics (an siRNA oligo cocktail) targeting multiple genes in the pathology to achieve much better therapeutic efficacy. This approach is based on two important facts: although inhibitory siRNA duplexes are sequence specific, all of them use an identical chemistry (dsRNA oligonucleotides); however, many human diseases are the result of overexpression of multiple endogenous and exogenous disease-causing genes. Using an siRNA oligo cocktail targeting multiple disease-causing genes represents an advantageous therapeutic approach with a synergistic effect. Nevertheless, even as a single agent, siRNA has tremendous therapeutic potential that will be realized when clinically feasible deliveries are developed.

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