RNA-Interference-Based Silencing of Mammalian Gene Expression

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1. Introduction

In the five years since its initial description, RNA interference (RNAi) has become a remarkably powerful method for the suppression of gene expression in a variety of organisms. As a technique that only requires short stretches of sequence information to generate loss-of-function alleles for any gene, it holds enormous promise in the functional analysis of genes found in the recently completed genome sequences. It has already become a standard tool in those organisms in which traditional gene-knockout analysis is not feasible. In mammals, in which traditional gene knockouts are labor-intensive, the advent of RNAi has generated a great deal of excitement in its application to drug-target identification and in its development as a potential therapeutic.

RNAi was first described as a startling phenomenon in which exogenously supplied doubled-stranded RNA (dsRNA) had potent and specific effects in reducing the expression of homologous endogenous genes.[1] Although it was immediately put to work as a genetic tool, several groups set out to uncover the novel molecular mechanism behind the phenomenon. The results of these studies, which include genetic evidence from *C. elegans* and biochemical evidence from a number of systems,[2] have provided a model in which RNAi occurs through a two-step mechanism (Figure 1). In the first step, a sequence-specific silencing factor composed of a 21 – 25-nucleotide (nt) short dsRNA is produced from the longer input dsRNA.[3, 4] Short dsRNAs of this size were originally isolated from *Arabidopsis* plants undergoing posttranscriptional silencing and were found to be complementary to both strands of the silenced gene.[5, 6] That similar short dsRNAs were also involved in RNAi was confirmed when, after isolation from cells undergoing silencing, they were sufficient to suppress gene expression in *Drosophila* S2 cells.[7–9] Since they appear to be a requisite component and are sufficient to trigger RNA interference, these small RNAs have been termed short interfering RNAs (siRNAs).

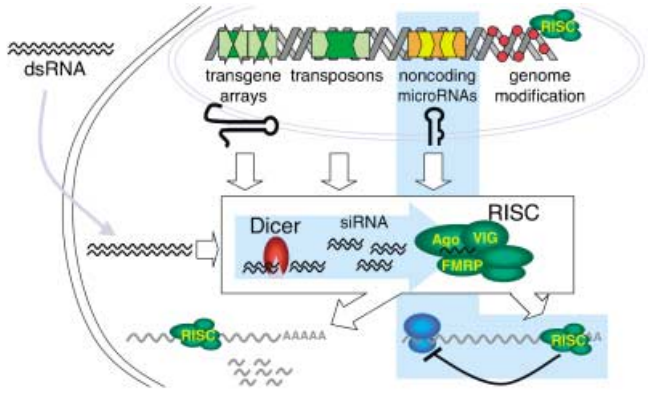

Figure 1. *Cellular roles of the RNA-interference (RNAi) machinery. In addition to processing exogenous dsRNA, the RNAi machinery is involved in the processing of other double-stranded RNA (dsRNA) species shown. Exogenous dsRNA and transgene-derived RNAs are processed into small interfering RNAs (siRNAs), which silence mRNA by degradation. In the case of the microRNA (miRNA) precursors, cognate targets are silenced by translational repression. The detailed mechanisms of transposon and chromatin silencing are unknown at present but involve transcription of repeat structures as an early step.*

The structure of siRNAs gave clues to the mechanism by which they were produced. Sequence analysis indicated that the siRNAs contained two nucleotide 3′-overhangs.[8] This structure suggested that the dsRNA was converted into siRNAs by an Rnase III family nuclease. Such an enzyme was first identified in *Drosophila* cells and subsequently found to exist in a number of eukaryotes capable of RNAi. In keeping with its function it was named Dicer.[10] Structurally, Dicer enzymes contain an amino-terminal DExH/DEAH RNA helicase domain, tandemly repeated RNase III catalytic domains, and a carboxy-terminal dsRNA-binding domain.[11, 12] They also contain a *piwi-argonaute-zwille* (PAZ) domain, which is specific to proteins involved in the RNAi pathway.

The second step of the RNAi mechanism was elucidated largely on the basis of biochemical evidence obtained in *Drosophila* cells.[8, 13–15] This work established that siRNA products of Dicer were incorporated into a multicomponent nuclease complex, termed RISC (RNA-induced silencing complex). This complex uses the sequence information contained within the siRNA as a specificity determinant in the identification and nucleolytic destruction within the region of homology of cognate mRNAs. Although the mechanism of this complex is still poorly understood, a number of its conserved protein components are now known. These include homologues of the Argonaute protein of *Arabidopsis*,[16] the fragile X mental-retardation protein and the Vasa intronic gene (VIG) protein.[17] Each of these has been implicated in some form of posttran-

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scriptional gene regulation; however, their roles within RISC are unknown. In general, the study of RISC is difficult. A number of *argonaute* gene family members are found in most species, which raises the possibility that different types of RISC complexes may exist within a cell, and the complex appears to play a part in multiple cellular processes (Figure 1).[18]

That dsRNA-induced silencing phenomena exist in a variety of evolutionarily diverse organisms[11, 19] suggested that the components of this system played a basic role in cellular biology. Since double-stranded RNA viruses and mobile genetic elements have the potential to form dsRNA structures and are virtually ubiquitous, it was thought that the RNAi pathway may have evolved early in eukaryotes as a cell-based immunity against genetic parasites such as viruses and transposable elements. In this light, the dsRNA-dependent silencing of transgenes in plants and endogenous genes in *C. elegans* were viewed as reprogrammed antiviral responses. It is now clear that this is only part of the story. The RNAi pathway is a central player in a variety of cellular processes related to the regulation of gene expression (Figure 1).

Endogenous small hairpin-shaped RNAs, which contain regions of dsRNA that are processed by the RNAi pathway, are now believed to be ubiquitous regulators of gene expression. The first examples of these, the *C. elegans lin-4* and *let-7* RNAs, were dubbed small temporal RNAs (stRNAs) owing to their role in the timing of developmental events.[20–23] Transcribed as short ($\sim$70 nt) hairpins, these RNAs are processed into a 21 – 22-nt mature form by the first step in the RNAi pathway and subsequently guide RISC complexes to the 3′ untranslated region (UTR) of target mRNAs through imperfect base-pairing interactions.[24–28] In the case of *lin-4* and *let-7*, expression of the targeted genes is inhibited at the level of protein synthesis, not mRNA destruction. This is now believed to be a common regulatory mechanism in eukaryotes, since hundreds of short hairpins collectively termed microRNAs (miRNAs) have been identified in *C. elegans*, *Drosophila*, mice, and humans.[29–33]

The RNAi machinery has also been shown to be involved in gene silencing that occurs in heterochromatin.[34] The large number of repeats and transposons found in eukaryotic genomes are frequently associated with centromeres within large regions of silent chromatin. In *Schizosaccharomyces pombe,* deletion of the genes encoding Argonaute, Dicer, or RNA-dependent RNA polymerase, which is responsible for the amplification of dsRNA in some species,[35, 36] abrogated the normal silencing of reporter constructs integrated within regions of centromeric heterochromatin. Deletion of these genes also impaired centromere function.[37] The precise mechanism of this process is a mystery. dsRNA transcribed from repeated sequences at the centromeres is processed by the RNAi machinery and in some way directs the methylation of histone 3 (H3) on lysine 9. Methylated K9 H3 binds heterochromatin protein 1, which in turn inhibits local transcription. Although surprising, this work echoes links between dsRNAs and silencing of plant transgenes by chromatin methylation of the homologous DNA region.[38, 39] These results are important in that they establish that the RNAi machinery is required for the pretranscriptional silencing of genes and the proper function of centromeric DNA. They have also sparked enormous interest in latent, noncoding dsRNA transcripts.

2. siRNAs in Mammalian Cells

From the outset, RNAi proved to be a useful genetic tool. It virtually revolutionized the genetics of *C. elegans*, enabling genome-scale RNAi-mediated gene-function analysis.[40–43] As mammals incorporate many of the same components of the RNAi pathway as *C. elegans*, it was hoped that it would have a similar effect on the genetics of mammals. Despite the mechanistic similarities, however, several hurdles related to the biology of mammals needed to be cleared before RNAi was proven to be an effective genetic technique in mammals.

The largest impediment to the implementation of RNAi in mammals is the physiological responses to dsRNA by these cells, which, when triggered, result in cell death. These include the induction of type I interferon (IFN) and the activation of two classes of IFN-induced enzymes: PKR, the dsRNA-dependent protein kinase, and 2′,5′-oligoadenylate synthetases, the products of which activate RNase L. As little as one molecule of dsRNA longer than 30 nt is sufficient to trigger these responses, eventually resulting in global inhibition of translation and apoptosis.[44–47] Although these responses are absent in some cell types, such as murine F9 and P19 embryonic carcinoma cell lines, which can therefore be subjected to long dsRNA-based gene silencing,[48–50] a more general solution to this problem was needed for RNAi to become universally useful in mammalian cells.

The key development in methodology for dsRNA-based silencing in mammalian cells was to employ dsRNAs that would fail to trigger the nonspecific dsRNA responses and yet still induce RNAi-type silencing.[9, 51] By using dsRNAs that mimicked siRNA duplexes produced by Dicer processing of long dsRNAs, Tuschl and co-workers were able to demonstrate specific gene silencing in a variety of mammalian cells.[9] Since these molecules are easily produced by chemical synthesis, this has rapidly become a standard technique for gene manipulation in mammalian cells. Applications of siRNAs have been reviewed extensively[52–56] and a variety of up-to-the-minute information is generally available online (e.g http://www.dharmacon.com/, http://www.ambion.com/techlib/resources/RNAi/index.html)

One interesting concept that has emerged from the extensive use of siRNAs in gene silencing is the variable susceptibility of target genes to siRNAs. The central event in RNAi-mediated gene silencing is the interaction of the siRNA contained in RISC with its complementary sequence within an mRNA. However, it appears that all interactions between siRNAs and target sequences are not equal. Not only do different genes respond differently to silencing, but considerable variability in the degree of suppression exists between target sequences within a single gene.[57, 58] It is thought that unknown intrinsic factors related to mRNA abundance, structure, translation rate, or other features of the RNAi mechanism are responsible. Further study of the mechanism of RNAi is required before we can accurately predict the suitability of a specific target sequence.

The target RNA-cleavage reaction guided by siRNAs is generally regarded as highly sequence-specific, requiring near identity between the siRNA and its cognate mRNA. For example, mismatches near the center of an siRNA duplex are most critical to target recognition and essentially abolish target RNA cleavage.[59] Mismatched bases near the ends of an siRNA contribute little to the specificity of target recognition. Some researchers have taken advantage of this to design target-allele-specific siRNAs.[60, 61] Despite these indications of the specificity, it is now clear that siRNAs can have effects on nontargeted sequences. Microarray studies have revealed that a single siRNA can affect the levels of a variety of messages within a cell that are not targeted.[62] These effects include the silencing of nontargeted genes containing as few as 11 contiguous nucleotides of identity to the siRNA. In a practical sense, it indicates that it is advisable to test several different siRNAs when silencing a gene of interest.

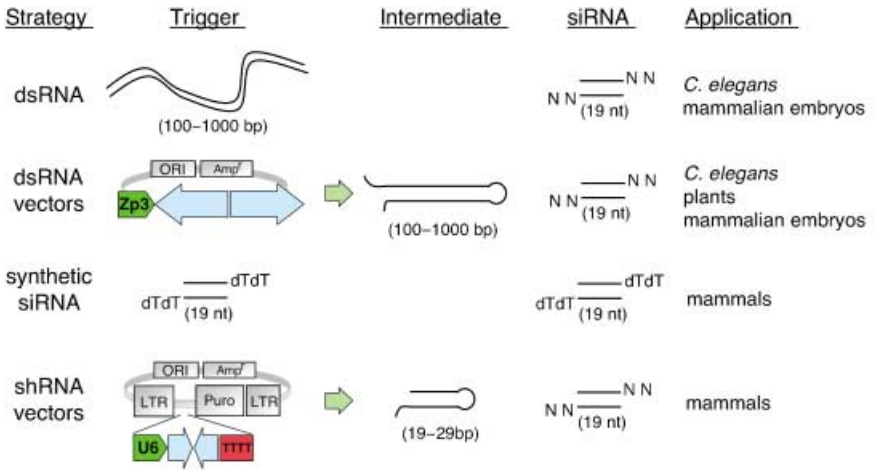

Figure 2. *RNA-interference delivery strategies. Four major methods of introducing RNAi triggers are listed with schematics and typical applications of each. A representative long dsRNA and short hairpin RNA (shRNA) vector are shown. The elements depicted in the shRNA vector are typically included to improve the gene-transfer efficiency and stability of the silencing effect. Note that a siRNA or siRNA-like molecule is generated in each approach.*

3. Stable Silencing

The development of siRNAs for use in cell-culture genetics is an enormous advance over the available technologies. Nevertheless, when compared to the power of RNAi in *C. elegans*, for example, mammalian siRNA-mediated silencing is somewhat limited. The first significant difference between RNAi in *C. elegans* and mammalian cells is that mammalian cells do not take up exogenously applied dsRNA efficiently. Mammalian cells must be subjected to cationic lipids or electroporation for siRNAs to be effective. Neither of these methods is particularly effective in vivo. Another major distinction between *C. elegans* and mammalian cells is that the RNAi response does not persist in mammalian cells. Mammalian cells lack the ability to amplify the RNAi response that worms have, and thus RNAi is limited to approximately 6 – 8 cell doublings.[63]

The issues of transfer efficiency and persistence of siRNAs in mammalian cells served as a catalyst for the development of stable RNAi-based silencing. In lower organisms, RNAi analysis of gene function was greatly improved by the in vivo expression of long dsRNA hairpins 500 – 1000 nt in length.[64–67] In each case, the production of siRNAs by in vivo transcription and endogenous Dicer cleavage improved both the delivery and duration of the silencing effect as compared to transient, dsRNA-based methods. Vector systems based on these ideas are now available for use in *C. elegans*,[65] *Drosophila*,[66] and plants.[68]

The development of stable long dsRNA hairpin-based expression systems in mammals, however, was not so straightforward. Long dsRNA is an effective approach for silencing genes in mammalian embryos,[69] and vectors have been constructed for this purpose (see Figure 2).[70] This approach is not universally applicable to cell lines. Although silencing by the use of long dsRNA has been accomplished in mouse embryonic stem (ES) cells and embryonic carcinoma cells,[70–73] the nonspecific effects of long (>30 nt) dsRNA expression in eliciting the interferon response in normal, differentiated somatic cell types[44][46] required a novel strategy for stable dsRNA-based silencing in these cells.

To overcome these problems, many groups turned to the endogenous short hairpin miRNAs as a model for expressing dsRNAs for silencing in cells.[58, 74–76] With short double-stranded stems, they were unlikely to trigger the nonspecific responses and were already known to be substrates of Dicer. Although the overall structure of miRNAs was retained, sequences within the stems of the encoded RNAs were engineered to be homologous to targeted gene sequences. In this way, silencing could be reprogrammed to specifically target any gene of choice. The similarity to miRNAs was somewhat superficial, however, as simple stem structures that were perfectly identical to the targeted gene and caused its degradation rather than translational arrest were found to be most effective.[76] Nevertheless, this approach largely solved the problems related to RNAi persistence and transfer efficiency in mammalian cells (see Figure 2).

To date, several groups have developed workable strategies for stable gene silencing in mammalian cells.[58, 74–77] In most cases, mammalian promoters are used to drive the expression of an interfering short hairpin RNA (shRNA) from DNA vectors introduced to cells by commonly used gene-transfer methods such as transfection or infection. Promoters using RNA polymerase III are employed in most cases, since this enzyme precisely initiates and terminates small, highly structured RNA transcripts[78] and is active in most, if not all, embryonic and somatic cell types. These include the mouse[79] and human U6-snRNA

promoters[76, 80, 81], the human RNase P (H1) RNA promoter[58, 74] and the human Val-tRNA promoter.[61] Although the production of shRNAs by using pol III is the most common approach, other strategies exist. These include expressing miRNA-like constructs from an RNA polymerase II (cytomegalovirus) CMV immediate-early promoter[75] and using separate U6 promoters to produce single-stranded 21-nt RNAs, which presumably anneal within the cell to form structures identical to siRNAs.[77]

For shRNAs, the available evidence suggests that construct design is relatively flexible. Double-stranded stems between 18 and 29 nt in length are roughly equivalent in efficacy.[82] Sequences of this length are long enough to serve as substrates for Dicer and contain unique silencing information, yet are still small enough to evade the PKR and interferon pathways. One of the strands of the stem structure should be complementary to the sense strand of the targeted mRNA. Whether it is the 5′ stem strand or the 3′ stem strand is not important.[58, 74, 76, 79–81] The sequence of the loop is also fairly unimportant. Loop structures between 3 and 9 nt in length work well, longer loops may be deleterious.[82] Target selection is poorly understood as is the case with siRNAs. The somewhat imprecise published guidelines with which to select hairpin target sites suggest a target sequence near the 5′ end of the gene with a GC content of approximately 50%. Many target sites that do not share these criteria are highly effective, including several cases in which the 3′ end of the gene was the best choice.[83, 84]

The many vagaries of short hairpin construct design have been incorporated in online design tools that simplify the entire process (http://katahdin.cshl.org:9331/RNAi/, http://jura.wi.mit.edu/bioc/siRNA/, http://www.dharmacon.com/, http://www.ambion.com/techlib/misc/siRNA finder.html). Once a target gene is selected, DNA oligonucleotides that encode shRNA corresponding to the gene are cloned downstream of a promoter into a vector suitable for gene transfer into mammalian cells. The power of shRNAs lies in their ability to continually silence targets after being stably transferred to host cells. For this reason, vectors that make use of gene-transfer methodologies that are inherently stable, such as retroviral integration, lentiviral integration, and adenoviral expression, are better choices for shRNA expression.

4. shRNA-Mediated Silencing in Animals

The stable suppression afforded by shRNAs has also been harnessed to affect the phenotypes of animals. The function of some genes, such as those that function in developmental, behavioral, and other complex processes, can only be studied in terms of the effect that a mutation has at the level of the organism. For this reason, classical mouse knockouts have been invaluable in investigating the function of many genes. Expression of an shRNA generates what is effectively a dominant loss-of-function mutation. That it is dominant avoids a major impediment of conventional knockout technologies, which require a significant investment of time and resources in the production of homozygous disruption alleles.

That stable RNAi could be used effectively to create loss-of-function alleles at the organism level in mammals was proven by using a modified hydrodynamic transfection method to deliver siRNAs and a luciferase reporter gene to the livers of adult mice.[85] Monitoring luciferase activity with quantitative whole-body imaging demonstrated that expression of a targeted luciferase reporter was specifically silenced to levels less than 5% of controls by a co-injected siRNA- or an shRNA-expression plasmid.

Recently, it has been demonstrated that short hairpin RNAs can be used to generate transgenic knockout animals.[86, 87] ES cells carrying shRNA vectors that targeted a ubiquitously expressed GFP reporter were used to generate transgenic mice that exhibited silencing in all tissues.[86] Suppression levels of up to 4% of untreated controls were observed and lasted for several weeks in F1 transgenic animals after introduction of the transgene. This approach has also been shown to be effective with endogenous targets.[87] An shRNA construct that effectively targets the murine DNA glycosylase-encoding *neil1* gene in ES cells was used to generate transgenic mouse lines. Animals that carried the *neil1*-targeted shRNA expression vector all displayed reduced levels of *neil1* mRNA and contained siRNAs corresponding to a Dicer-processed form of the shRNA. Consistent with a role for *neil1* in DNA repair, cells from these mice exhibited increased sensitivity to ionizing radiation.

Several groups have now reported improved vector systems for the construction of shRNA-based knockdown animals.[88–90] These vectors are based on self-inactivating lentiviruses, which are less susceptible to silencing during mouse development and are ideally suited for the generation of transgenic animals through infection of embryonic stem cells or single-cell embryos.[91] In addition to an shRNA expression cassette, most express EGFP as a reporter that allows infected cells to be selected by flow cytometry. A variety of reporter and endogenous genes have been silenced in mice using these systems.

A general issue with RNAi-based approaches is whether the reduction of expression, which typically approaches but does not reach 100% reduction in expression, is sufficient to generate an obvious phenotype. Although it was originally hoped that RNAi in transgenic animals would substitute for the classical generation of knockouts, it seems likely that in some instances RNAi will prove to be a poor substitute for the complete disruption of a gene. Nevertheless, it is now clear that hairpins that are less than completely effective may be equally valuable as an investigation tool. Incomplete loss-of-function mutations in genes essential for viability are, in general, much more useful than nulls. This is true of genes involved in development where classical gene knockouts are of limited use as they eliminate gene function universally in the embryo. Even in genes that are not lethal, hairpins that do not completely silence a targeted gene are useful since they can give rise to subtle phenotypes that aid in determining the function of a gene in a given process. In a study with retroviral shRNA constructs that had intrinsic differences in their ability to silence the targeted mouse p53 gene, different hairpins produced phenotypes of varying severity at both the cellular and organism levels.[92] *p53* shRNAs that drastically reduced levels of the tumor-suppressor protein led to the formation of aggressive tumors and premature death of mice. Relatively ineffective *p53* shRNAs had minimal effects on

p53 levels and resulted in animals with a mild form of the disease with no effects on mortality. The utility of shRNAs that do not completely silence their target, coupled with the increased ease of generating mutant animals represent significant advances in the methodologies for elucidating gene function in vivo.

5. Summary and Outlook

Owing to the robust nature of the process, RNAi-based gene silencing is certain to be a valuable technique for the foreseeable future. That it is effective in vitro and in vivo suggests that the limits to its utility are only in the types of phenotypes and assays that apply to mammalian cells. The rugged nature of the approach derives in large part from the fact that RNAi, as opposed to antisense, makes use of a collection of cellular enzymes that has been honed by millions of years of evolution for the express purpose of gene silencing.

Although highly useful already, a number of improvements to the current technologies are likely to emerge in the near future (see Figure 3). Inducible hairpin promoters that express shRNAs only in response to small molecule inducers or in specific tissue types would be valuable in the analysis of genes that are essential, have multiple roles in development, or that are related to behavior. Vectors that express several silencing constructs simultaneously would improve combinatorial silencing.[82] Since RNAi acts in a dominant fashion, multiply mutant cells and animals can be generated in the time that it takes to generate a single heterozygous founder by conventional methods.

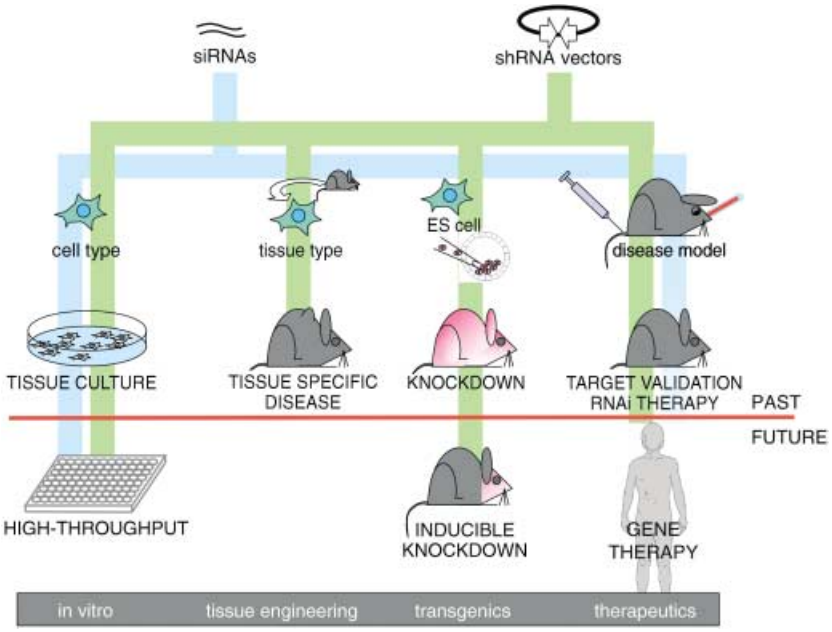

Figure 3. *Applications of RNA-interference-based silencing in mammals. Schematic summary of current (above red line) and probable future (below red line) uses of RNA interference. siRNAs are typically used in cell cultures, but have also been used in some therapeutic studies in mice. shRNA vectors are especially useful in the generation of stable, hypomorphic alleles of genes in tissue culture cells, in specific cell types within mice, and in entire animals. High-throughput, inducible knockouts and gene therapy are under development at the time of this writing.*

Perhaps the most pertinent application of RNAi in mammalian cells is directly related to the fact that its discovery has coincided with the appearance of thousands of genes of unknown function in the mammalian genomic sequencing project databases. Since RNAi requires only short DNA sequences to manufacture potent reagents to knockdown gene expression, the sequence databases provide all the information required for genome-wide gene-function analysis projects. Arrayed libraries of siRNAs or hairpins that target each ORF in a mammalian genome are being constructed for use in phenotype-based, mid- to high-throughput screens in vitro and in vivo.[2, 93, 94] The hope is that the role of any gene product in a given biological process can be tested without extensively developed reagents or presuppositions of its function.

Finally, RNAi may have a future in the direct treatment of disease.[94–104] In contrast to standard gene therapy, which normally relies on the ectopic expression of proteins, RNAi can diminish the effects of deleterious gain-of-function mutant genes or the genes of infectious pathogens. Evidence for the potential of RNAi as a therapeutic has been demonstrated by a number of groups.[105–107] As with any gene-therapy approach, however, issues related to delivery and safety are likely to pose major obstacles. Until these are overcome; RNAi will remain one of the most important tools for target discovery and validation in the development of small-molecule therapeutics.

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