Gene Therapy (2004) 11, 1241–1248

www.nature.com/gt

REVIEW

Gene Therapy Progress and Prospects. Downregulating gene expression: the impact of RNA interference

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The control and maintenance of gene expression is critical for cell development and differentiation. Over the last 2 years, our understanding of the role of RNA as a regulator of gene expression has significantly increased. Small RNA molecules are key elements of a machinery that trigger chromosomal modifications, post-transcriptional gene silencing and protein translational blockade depending on the source, the RNA and the nature of the interaction with the target nucleic acid. Currently, the best characterized of this group of RNA-mediated gene regulation pathways is the post-transcriptional gene silencing mechanism known as RNA interference. RNAi is triggered by double-stranded RNA (dsRNA), which induces the formation of a ribonucleoprotein complex that mediates sequence-specific cleavage of the transcript cognate with the input dsRNA. RNAi has been adapted as a functional genomics tool and it has potential as a therapeutic approach. This review will summarize our current understanding of the RNAi mechanism and the various applications of RNAi-based technologies.
Gene Therapy (2004) **11,** 1241–1248. doi:10.1038/sj.gt.3302324

Keywords: *RNA interference (RNAi); gene silencing; short (small) interfering RNAs (siRNAs)*

In brief

Progress

- RNAi is a naturally occurring gene silencing mechanism.
- The RNAi mechanism has a role in controlling endogenous gene expression.
- RNAi can be used as functional genomics tool.
- The siRNA sequence is important for RNAi in mammalian cells.
- Optimization of the delivery of RNAi effectors is required.
- RNAi-based therapeutic applications are being addressed.

Prospects

- Our understanding of the role of RNAi and its associated pathways will improve.
- Methods that exploit additional aspects of the RNAi mechanism will be developed.
- Routine use of RNAi to determine gene function and expansion of large-scale RNAi screens.
- Additional parameters that determine efficacy and specificity of siRNA/shRNAs will become available and will be better understood.
- The delivery and stability of siRNAs in cell culture and *in vivo* will need to be improved.
- More detailed investigation of RNAi against clinically relevant gene targets in appropriate model systems will be performed.

Downregulation of gene expression with RNAi

Most gene therapy approaches have focused on a gain of function to alter the phenotype of a cell, but there is increasing interest in developing therapies based on inhibition of function using nucleic acid molecules. The delay in development of nucleic acid-based approaches to the inhibition of protein function was perhaps to be expected in that any methodology based on downregulating gene expression must perform at least as well as a small molecule drug that inhibits the same target or inhibit a target not amenable to interaction with a standard antagonist. Technologies aiming to inhibit gene expression have usually involved complex genetic manipulations, which disrupt the genomic sequence or exploit interactions with the encoding transcript, leading to either a block in translation principally through the

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use of antisense molecules or a degradation of the mRNA using ribozymes. These methodologies remain promising, but it has proven difficult to adapt them as broadly applicable functional genomic and therapeutic tools. Although elements of these established approaches are based on naturally occurring interactions that modulate gene expression, it is still unclear to what extent any of these processes act directly or indirectly to control endogenous gene expression. In contrast, in recent years, we have significantly increased our understanding of a group of related, naturally occurring mechanisms that inhibit gene expression at a post-transcriptional level. Known as post-transcriptional gene silencing (PTGS) in plants and fungi, and RNA interference (RNAi) in animals, the identification and characterization of these pathways has begun an important new phase in the development of technologies based on downregulating gene expression. These technologies, in addition to traditional antisense and ribozyme-based strategies, have the potential to assist in improving our understanding of gene function, of improving methods to identify and validate drug targets and potentially as a means of therapy.

RNAi is a naturally occurring gene silencing mechanism

Until recently the dominant roles for RNA have been as an encoder of genetic information, either directly as in retroviruses or as an intermediate through messenger RNA (mRNA), and as key components of the protein translational process through the activities of transfer and ribosomal RNA. However, it is becoming increasingly evident that RNA also has a direct role in processes that regulate gene expression. The term RNAi was first coined to describe the sequence-specific, post-transcriptional inhibition of gene expression induced by the introduction of a cognate double-stranded RNA (dsRNA) molecule into the nematode worm *Caenorhabditis elegans.* RNAi, triggered by dsRNA, was subsequently identified in a broad range of invertebrate species including *Drosophila*. Interestingly, a post-transcriptionally mediated silencing of gene expression had previously been observed in plants and fungi, where the cloning of multiple copies of a transgene homologous to an endogenous gene resulted in the suppression of protein production from both the transgene and the endogenous gene. This effect, known as PTGS, is also triggered by cytoplasmically replicating RNA viruses in plants. Studies, predominately in plants, fungi, *C. elegans*, *Drosophila* and most recently in mammalian cells have now shown that dsRNA is a key trigger of gene silencing processes in these, and probably all species, and that these mechanisms involve the formation of a ribonucleic protein complex termed the RNA-induced silencing complex (RISC) (Figure 1).

The central RISC protein is a member of the Argonaute family of proteins (*Drosophila* Ago2, human Ago2/EIF2C).[1] Additional proteins associated with human RISC include Gemin3 and 4.[2–5] In *Drosophila*, the Vasa intronic gene product (a p68 RNA helicase homolog) and the fragile X mental retardation protein are associated with RISC.[6,7] The RNA component of RISC that interacts with the target transcript is a small species ~20–25 nucleotides (nts) (predominately 21–22 nts) of single-stranded RNA (ssRNA).[2,4,8–10] Exogenous dsRNA introduced into invertebrate cells are cleaved by an RNase III enzyme called Dicer into a species of RNA molecule called small (or short) interfering RNAs (siRNAs).[11,12] SiRNAs are initially RNA duplexes of 20–25 nts in length with 3′ two or three nucleotide overhangs and a 5′ phosphate.[12,13] The duplex siRNA is unwound and RISC plus the single-stranded siRNA species base pair with the target RNA. Interestingly, a bias in the formation of RISC complexes with a particular RNA strand has been noted. This bias is thought to reflect how the siRNAs are processed, unwound, and loaded into RISC, with the stability of the nucleotide interactions within the siRNA being a critical determinator.[9,10,14] The target RNA is cleaved at a position ~10 nucleotides from 5′ end of the antisense siRNA sequence.[15] A candidate ribonuclease, with homology to the Tudor staphylococcal nuclease, has been identified as associated with RNAi but additional work is required to confirm that this is the RNase responsible for RNAi-directed cleavage.[16] In mammalian cells, with the exception of embryonic derived cells, siRNAs must be introduced into or expressed directly in cells to minimize the triggering of interferon-associated proteins that mediate a variety of nonspecific responses that alter gene expression.[17–19] The central RNAi pathway involving siRNAs and RISC appears to exist in all organisms studied to date; however, there are some aspects of the RNAi silencing mechanism that are specific to certain species. For example, in plants, *Neurospora*, and *C. elegans,* there is evidence for an amplification and spread of the gene silencing effect. The amplification of RNAi has been best characterized in *C. elegans*, where there is evidence that an RNA-dependent polymerase uses the mRNA target and the antisense strand of the siRNA to prime production of further siRNAs.[20] Also in *C. elegans*, a protein, SID-1, has been identified that facilities the uptake of dsRNA, suggesting that *C. elegans* actively uses dsRNA to respond to external triggers.[21,22] There is no evidence for amplification or spread of the gene silencing effect seen in mammalian cells.

RNAi mechanism has a role in controlling endogenous gene expression

Although siRNAs produced from exogenous sources, for example, viruses, transgenes or directly administered dsRNA or siRNAs, were the first RNA molecules to be shown to interact with the protein components of the RNAi machinery, there is increasing evidence that endogenously expressed small RNA molecules interact with components of the RNAi pathway to induce regulation of gene expression. In *Schizosaccharomyces pombe*, siRNAs produced by transcriptional active repeat elements within heterochromatin regions are thought to act to trigger histone modifications, which trigger hetrochromatin formation and stability.[23–27] For example, knockouts of components of the RNAi pathway including Dicer and the *S. Pombe* homolog of *Ago2 (Ago1)* lead to transcription from previously silent regions.[23] There is also evidence linking RNAi with suppression of aberrant expression from transposons.[28–30] Another link between RNAi and the control of endogenous gene expression is the processing, by Dicer, of micro-RNAs

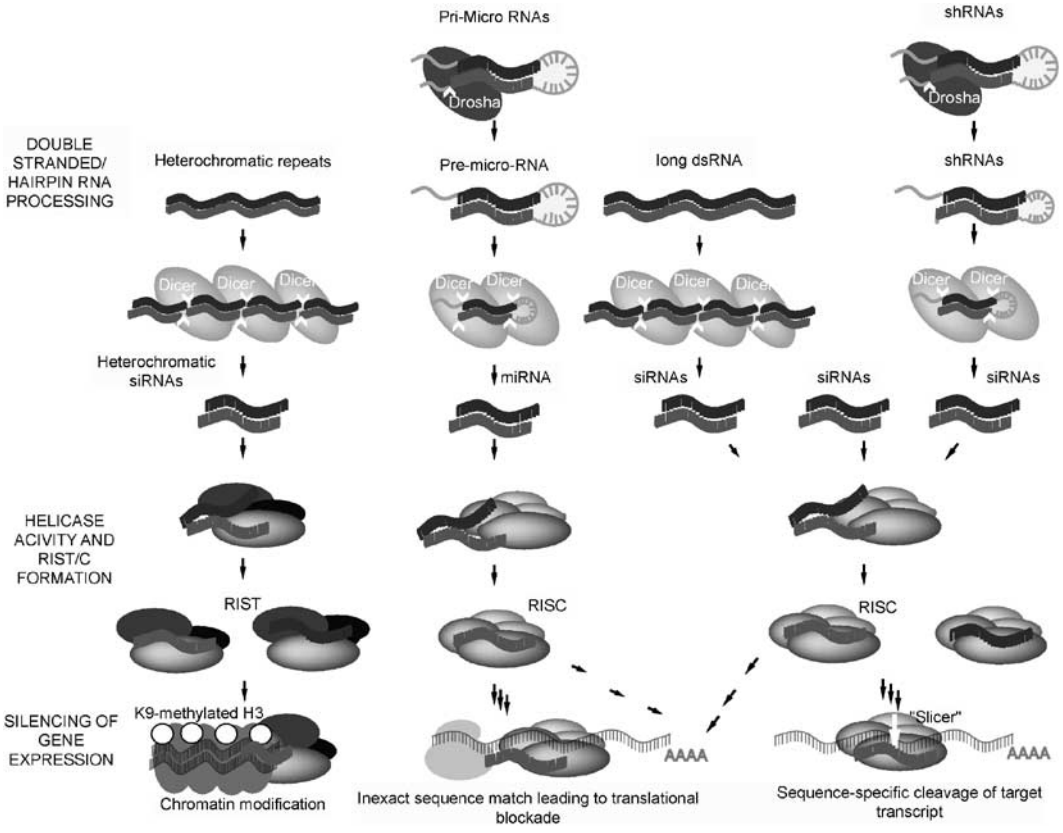

Figure 1 *RNAi and the mediation of gene silencing. RNAi can induce gene silencing in a variety of ways depending on the structure of the initiating RNA molecule, the protein(s) recruited that form the RNA–protein complex facilitating interaction of the small RNA molecule with its target and the sequence alignment of the small RNA molecule with its target molecule. Precursor microRNAs (pre-miRNAs), which are exported from the nucleus to the cytoplasm, are initially processed by the RNase III enzyme Drosha from endogenously expressed primary micro-RNAs (pri-microRNAs). Short hairpin RNAs expressed from RNA polymerase II or III promoters are also thought to be processed by Drosha. Processing by Dicer releases small RNA molecules (micro RNAs (miRNAs), or small interfering RNAs (siRNAs) that are unwound and incorporated into an RNA–protein complex. The end of the small RNA molecule with the lowest free energy is the end from which the small RNA molecules are preferentially unwound. The RNA–protein complex associated with heterochromatin formation in Schizosaccharomyces pombe, termed the RNA-induced initiation of transcriptional gene silencing (RIST) complex, contains S. pombe Ago1 and Chp1, a protein required for methylation of histone H3-K9.[27] The human RISC required for miRNA- or siRNA-mediated protein translational blockade or transcript cleavage contains EIF2C (human Ago2) Gemin3 and 4.[1–5] Sequence alignment between the small RNA molecule and the target transcript determines whether protein translational blockade or transcript cleavage is mediated.*

(miRNAs), 21–23 nt ssRNA molecules, from pre-miRNAs (70–80 nts) that form hairpin loop structures.[31–34] Several hundreds of micro-RNAs have been identified in a wide range of species, some of which are associated with the regulation of developmentally essential genes, which may explain, at least in part, why transgenic disruption of the mouse Dicer gene results in an embryonic lethal phenotype.[35] How miRNAs control gene expression is still being elucidated. In the best-characterized interaction of miRNAs and their target mRNAs, the sequence alignment between miRNAs and the transcript involves an inexact sequence match between the two RNA species, which appears to lead to a structural perturbation of the mRNA that blocks translation. However, in plants, there is an exact match between the miRNA and the putative target sequence, suggesting that both an siRNA-like specific cleavage or an miRNA-like protein translational blockade can occur. Model systems suggest that siRNAs and miRNAs can act interchangeable as only sequence alignment differentiates between the two, an important concern when considering if siRNAs can generate off-target effects when used to inhibit gene function, see below.[36,37] The structure of miRNAs has been exploited as the basis of another group of molecules that can trigger RNAi, known as short hairpin RNAs or shRNAs. ShRNAs consist of a ssRNA molecule (synthesized, *in vitro* transcribed or expressed) that form a stem loop structure where the stem corresponds to the siRNA.[38–41]

RNAi can be used as a functional genomics tool

The principal application, to date, for RNAi-based technologies has been to elucidate gene function mainly focusing on establishing RNAi knockdowns of previously characterized genes to understand further the functional role of these genes. Since the first reports of siRNA-mediated RNAi in mammalian cells in 2001, RNAi against transcripts encoding proteins involved in a wide range of cellular processes have been reported. More complex functional genomics applications of RNAi knockdowns include complete pathway delineation and the translation of associative bioinformatic, positional cloning and cDNA microarray data into functional data as the only requirement for generating the RNAi resources is transcript sequence information.[42,43] One advantage of RNAi knock-

downs is that multiple genes can be studied simultaneously or consecutively; this application is particularly important as it should allow the analysis of genes, where functional redundancy is an issue, and so where the knockout of one gene alone has generated no useful phenotype; however, the RNAi machinery can be saturated and so there will probably be a limit to the number of different genes that can be targeted in a cell at one time. To enable RNAi functional genomic analysis to be conducted on a large scale, RNAi libraries have now been created corresponding to either a significant proportion, in the case of the human genome, or the whole genome of *C. elegans* and *Drosophila*.[44–48] New platforms are also being developed to facilitate this type of RNAi genomic scale analysis on a high throughput basis.[49,50]

Another feature of RNAi knockdowns is that there is a variation in the degree of inhibition mediated by different siRNA sequences. On the surface, this may appear to be a disadvantage as this requires extensive bioinformatic and experimental analysis to determine the most effective siRNA against a particular target, but it actually has the advantage of being able to modulate gene expression to produce different phenotypes. For example, three different shRNAs against the tumor suppressor gene *Trp53* have been used to develop a series of hematopoietic stem cell lines expressing differing levels of Trp53. When these HSC lines were introduced as xenografts into an animal model, significant phenotypic differences were observed.[51] This example points to another application of RNAi in the generation of new model systems including cell lines and transgenic animals. Transgenic mice exhibiting a knockdown phenotype as a result of the expression of an shRNA against a particular target gene have been created using various approaches including lentiviral vector transduction of mouse ES cells.[52–56] An alternative way in which viral delivery of shRNAs can be used to generate a disease model is by localized inhibition of a gene; for example, adeno-associated viral delivery of shRNAs corresponding to the dopamine synthesis enzyme tyrosine hydroxylase into the mid-brain neurons of adult mice has been used to generate a model of Parkinson disease.[57] Adaptation of expression vectors to use inducible or tissue-specific promoters to drive expression of the shRNA should enable investigation of the effect of downregulating gene expression in a particular context, including the study of developmentally essential genes. Finally, one of the most critical applications for RNAi in mammalian cells is as a tool for validating the targets of small drug molecules and identification of new drug targets. Small molecule drugs or the trigger of RNAi against that target can then be developed or a molecule that triggers RNAi could also be exploited as a therapeutic agent. However, there are specific issues related to inducing RNAi in mammalian cells that first need to be considered.

siRNA sequence is important for RNAi in mammalian cells

Sequence alignment between the RNA component of RISC (eg single-stranded siRNA, or miRNA) and its target transcript is key to determining the mode, efficacy and specificity of gene silencing. RNAi performed in invertebrates (principally *C. elegans* and *Drosophila*) utilizes large dsRNA molecules, usually 100–500 bp, which exploit the endogenous activity of Dicer to generate siRNAs within the cell. Dicer generates a pool of siRNAs from the input dsRNA, which will have an exact match to the target mRNA, but which could also include siRNAs that potentially could have sufficient homology with several transcripts to trigger an miRNA-like gene silencing event. In fact, little or no nonspecific effects have been seen in these species, suggesting that a selection for the most specific and effective siRNAs within the pool may occur perhaps during processing of the dsRNA or perhaps during the formation of RISC. Pools of siRNAs, for transfection into mammalian cells, can be produced *in vitro* by endonuclease digestion of long dsRNA molecules using either Dicer or bacterial RNase III, but this methodology has been used less frequently than that of synthetic siRNAs and shRNAs, and off-target, miRNA-like responses have been reported.[58–61] Recently, restriction endonuclease digestion of cDNA (including complex cDNA libraries) has been used to generate libraries of shRNAs that contain pools of shRNAs against the same target. Initial studies show that multiple shRNAs generated in this manner can be relatively quickly screened to identify those mediating a significant RNAi effect.[62,63]

The use of synthetic siRNAs and expressed shRNAs to trigger RNAi in mammalian cells requires a preselection of the sequence. The choice of siRNA sequence needs to consider a number of issues, including avoiding nontarget sequence homologies of both strands of the input siRNA, sequence parameters that influence the effectiveness of a particular siRNA, and the efficiency with which the siRNA is unwound and interacts with the RISC protein complex. In addition, sequence constraints that allow expression of the siRNA as an shRNA may influence the efficiency with which it is processed to form an effective siRNA. The basic structure of synthetic siRNAs used for gene silencing in mammalian cells mimics that of naturally occurring siRNAs isolated from *Drosophila* extracts and are thus 21 or 22 nts in length with 3′, usually two nucleotide overhangs, which for ease of synthesis often consist of 2′ deoxynucleotides; however, this feature does not reflect a requirement of the mechanism. Initial siRNA/shRNA sequence design algorithms were developed using limited experimental data primarily based on experimentation in *Drosophila* cells rather than mammalian cells. Algorithms using additional data generated in mammalian cells are in development and should increase the likelihood of generating an effective siRNA/shRNA sequence that inhibits gene expression by a predictable amount.[10,64] One aspect of the choice of sequence that has seen much recent attention is the evidence that there is a bias in the formation of RISC complexes, where the incorporated single strand of the siRNA has a lower free energy at the 5′ end when compared with the rest of the siRNA molecule (ie has a higher AU content over approximately the first 2–5 nucleotides than the rest of the molecule).[10,65] It is through that RISC attempts to unwind the siRNA from both direction but the least stable end will be favored, and thus if designed appropriately, it should be possible to significantly increase the probability that the antisense strand corresponding to the target transcript will be incorporated into RISC rather than the sense,

thereby reducing the possibility of off-target effects induced by the sense strand. Another reduction in the free energy levels within the siRNA is seen around the cleavage site region. It is assumed that the relatively high free energy values obtained for the other regions of the molecule are required to ensure specific interaction with the target transcript. Obviously, the choice of a particular siRNA sequence must also consider alternative or mutant transcript variants, which may need to be targeted independently or simultaneously.

Validation of the RNAi effect in mammalian cells is important

The most cited application of RNAi in mammalian cells is the inference of gene function following reduction in the levels of the corresponding protein. RNAi has proved highly successful in this role; however, it is important to confirm that any inferred functional effect is due to RNAi. These types of verification experiments are also relevant to studies investigating the therapeutic potential of RNAi against a particular target, and in many ways more so, since ensuring the specificity and quantifying the efficacy of the particular siRNA or shRNA against a clinically relevant target transcript is essential in justifying its further development. There are a variety of means by which functional data derived from RNAi knockdowns can be validated and guidelines have been suggested.[66] Both a decrease in the RNA and protein levels of the target gene should be observed to establish siRNA-mediated RNA, and siRNAs of different sequences against the same transcript should be assessed in independent experiments; ideally, a dose-dependent effect should also be seen for each siRNA. The use of siRNAs against genes thought to be involved in a pathway upstream or downstream of the principal target gene can also be used to confirm novel phenotypic findings and it should be possible to rescue the functional phenotype induced by RNAi by expression of a transcript resistant to the siRNA under study.[67] The level of RNAi knockdown may also be critical for interpretation of downstream effects, as will the time at which a particular phenotypic effect is assessed. A limited number of studies have directly compared the effectiveness of RNAi-induced silencing and antisense-based technologies. Vickers and co-workers suggested that the degree of inhibition was similar when highly optimized antisense oligonucleotides and siRNAs were used, although others have reported that RNAi is more potent than antisense inhibition, as significantly less nucleic acid is required and less optimization is necessary.[68–70] Interestingly, one group has suggested that the combined use of antisense oligonucleotides and siRNAs may enhance their ability to inhibit expression.[71] While RNAi appears to be easy to induce, critical analysis of RNAi derived phenotypic data should not be overlooked.

Nonspecific effects of RNAi need to be carefully assessed in mammalian cells

The exposure of cells to any exogenous molecule, including nucleic acids and its delivery agent, has the potential to perturb normal cellular function and so any manipulation to alter the function of a particular gene can only aim to maximize the ratio of specific to nonspecific effects. The large number of reports utilizing RNAi in mammalian cells that have been published in the last 3 years suggests that in most cases investigators are generating appropriate results that reflect a level of specificity, which allows inference of gene function. However, there are some specificity issues unique to the induction of RNAi in mammalian cells that have to be considered. First, mammalian cells have nonspecific dsRNA-triggered responses, primarily mediated through interferon-associated pathways that are absent in invertebrates and plants. Only limited studies have attempted to address the relationship between siRNA-mediated RNAi and other dsRNA responses, but these studies have suggested that siRNAs can trigger a nonspecific effect upregulating interferon responsive genes, an effect that is independent of their ability to mediate sequence-specific inhibition of gene expression, and that shRNA expression from vectors can upregulate the interferon response gene 2′5′-oligoadenylate synthetase.[72,73] Recently, it has been shown that characteristics of the RNA polymerase III promoter U6 may contribute to the nonspecific effects seen following the expression of shRNAs from this promoter (Pebernard, 2004 #522). Optimization of transfection conditions, particularly the use of conditions that minimize the amount of siRNA (and also the transfection agent) may be critical to reducing these more general cellular responses. A second issue is that of using siRNAs; the RNAi machinery is accessed downstream of Dicer, so that processing steps, which may help determine specificity and efficacy of RNAi when Dicer acts against a larger dsRNA molecule, are not utilized. One way of influencing this is by careful choice of the siRNA sequence to ensure that the antisense sequence against the target of interest is preferentially maintained and that homology to any other target is minimized,thereby reducing the probability that the siRNA sequence can act as an miRNA.[10] However, it is unclear at this time what the minimum level of homology required between the siRNA and the target to decrease gene expression is, but it has been reported that matches of as few as 11 consecutive nucleotides can affect the RNA levels of a nontargeted transcript.[74] Although these crosstranscript effects have been noted using siRNAs, siRNA-mediated RNAi in mammalian cells can be sufficiently specific to allow for the targeting of allelic-specific transcripts that differ by as little as one nucleotide if the mismatch is positioned towards the middle of the siRNA. This has enabled groups to suggest strategies for developing RNAi triggers that specifically target a mutant allele, an attractive approach for treating some dominant genetic disorders.[75]

Optimization of vectors for the delivery of RNAi effectors is required

Currently, RNAi-based technologies make use of established methods for the delivery of nucleic acid into mammalian cells. Chemically synthesized siRNAs formed from annealed complementary RNA oligonucleotides are most effectively introduced into cells using cationic liposomes or electroporation, although protocols

and transfection agents optimized for the delivery of plasmid DNA to the nucleus usually need to be adapted, as siRNAs need only be delivered to the cytoplasm. The transient nature of the inhibition of gene expression in mammalian cells by siRNAs, which at an RNA level usually peaks about 48 post-transfection, can be overcome by expressing shRNAs from plasmids harboring a selectable marker. Short hairpin transcripts are usually expressed from an RNA polymerase III promoter, although RNA polymerase II-driven shRNA constructs have also been constructed. Plasmid and viral (retrovirus, lentivirus adeno-associated virus and adenovirus)-based vector systems have been used to express shRNAs. However, it is important to consider the possibility that these vector systems may also generate effects on gene expression independent of the expressed siRNA sequence.[41,53,55,76] All of these nucleic acid delivery systems have the potential to be used to develop RNAi as a therapeutic approach.

RNAi-based therapeutic application are being addressed

The treatment-related activity of most small molecule drugs is to inhibit the function of its target molecule in as specific a manner as possible. Turning RNAi from a functional genomics tool into a therapeutic tool thus seems a logical outcome of studies using RNAi. However, developing RNAi-based therapeutics faces many of the same challenges, which the antisense and gene therapy fields have faced for the last decade, in translating cell culture data into positive outcomes in preclinical *in vivo* models and in the clinic, namely delivery to the right cell type in the right organ, at the right time with no or minimal nonspecific, including immunological effects. A large number of disease relevant target transcripts have been subjected to RNAi, including genes associated with cancer (eg *K-ras*),[41] viral infections (eg HIV-1)[77] and single gene disorders (eg spinocerebellar ataxia type 3), primarily in cell culture models using both siRNAs and expressed shRNAs.[75] In some cases, RNAi-induced sequence-specific downregulation in gene expression has been proven using all of the criteria described above. However, in most studies, data are still relatively preliminary and true preclinical studies are probably still some time away. On a positive note, RNAi has been induced *in vivo* in adult mice using synthetic siRNAs and shRNAs expressed from plasmid and viral vectors.[76,78,79] In these initial studies, high-pressure tail-vein injections of mice were used to administer either an expression plasmid and an siRNA or an siRNA alone.[78,79] Sequence-specific silencing was seen in tissues (principally the liver) harvested from the injected mice with up to about 80% inhibition being observed. The first proof of principle *in vivo* delivery of an shRNA was achieved using an adenoviral vector in the brain of mice and the liver.[76] More recently, studies have attempted to use RNAi to modulate a disease process in a variety of model system, for example, several groups have downregulated apoptosis-associated genes as a way of protecting liver cells from liver fibrosis and allogenic hepatocyte transplatation.[80–82] These proof of principle experiments are encouraging, further modifications of siRNAs to enhance *in vivo* delivery and the development of optimized vector systems specifically designed for the expression of shRNAs could enhance the feasibility of applying RNAi as a therapeutic tool.

Summary

The expanding role of noncoding RNAs in controlling gene expression has opened up a whole new understanding of epigenetic mechanisms. These basic biology studies have lead to the very rapid development of methods that exploit at least one of these mechanisms, RNAi. RNAi is being used to understand gene-function relationships and should significantly speed the discovery and validation of new therapeutic targets for small molecule drugs, as well as the development of the molecular triggers of RNAi as therapeutic agents in their own right.

Prospects

RNAi has been used to inhibit gene expression from clinically relevant transcripts, and studies in suitable preclinical models are beginning. These studies will need to address the specificity and efficacy of any effect induced by the molecule triggering RNAi. The principal issue turning RNAi from an effective functional genomics tool into a therapy remains one of delivery. RNAi primarily acts within the cytoplasmic compartment, which is easier to access using nonviral methods than the nucleus, but ensuring efficient uptake and long-term stability *in vivo* in disease relevant tissues is still likely to be difficult. Viral delivery of shRNAs is effective *in vivo* but clinical targets will have to be found, where the inhibition of the target gene will have a clinically beneficial outcome that outweighs any of the established risks associated with these vectors, in particular, problems related to the repeated administration of viral vectors. Transcripts related to tumor progression and viral replication may be the most appropriate first targets, although targeting just one gene may have only a minimal effect on tumor growth and the high rate of mutation of viruses could be a problem. While issues related to the specificity of RNAi remain, caution should also be exercised. However, the prospects for overcoming these are good as we improve our understanding of the RNAi mechanism.

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