

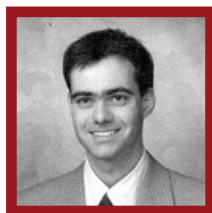
POTENTIAL APPLICATIONS OF RNAi:

- Cancer
- Infectious and neurodegenerative diseases
- Macular degeneration
- Septic shock



RNAi AND DISEASE BIOLOGY

RNAi has been hailed as one of the most exciting discoveries in biology and has proved to be a powerful tool in the field of functional genomics. Although it has a huge role to play in the field of therapeutics, there are many issues still to be addressed, writes Oliver C Steinbach, head of functional genomics, Altana Pharma.

**Author**

Since 2002, Oliver Steinbach has served as the director of functional cloning for the Altana Research Institute. Specialising in the application of genomics and proteomics technology with underlying automation technology and bioinformatics, he manages the discovery and validation of novel pharmaceutical targets used in the treatment of inflammation, gastroenterology and oncology.

Molecular biology entered a new era when ncRNA were found to play a role in aspects of cellular regulation (gene expression, development, chromatin remodeling and possible rebuilding of chromosomal DNA). Diverse phenomenon quelling (in fungi), RNA interference (in animals) and cosuppression or posttranscriptional gene silencing (in plants) all follow the same fundamental mechanism involving ncRNAs, now called RNAi. Here, non-coding dsRNA eventually degrades or inhibits the translation of the target RNA, causing a knock-down of gene expression, instead of the permanent mutational alteration of the gene, known as 'knockout'.

The mechanism of RNAi has been uncovered in some detail. Long dsRNAs are processed into 20-25bp siRNAs by an RNaseIII-like enzyme called Dicer. The siRNA strands are then unwound by an ATP-dependent helicase. Each siRNA strand associates in a riboprotein complex containing an endoribonuclease-complex RISC. The siRNA strands then bind to the complementary target RNA molecules, causing their degradation by the RISC.

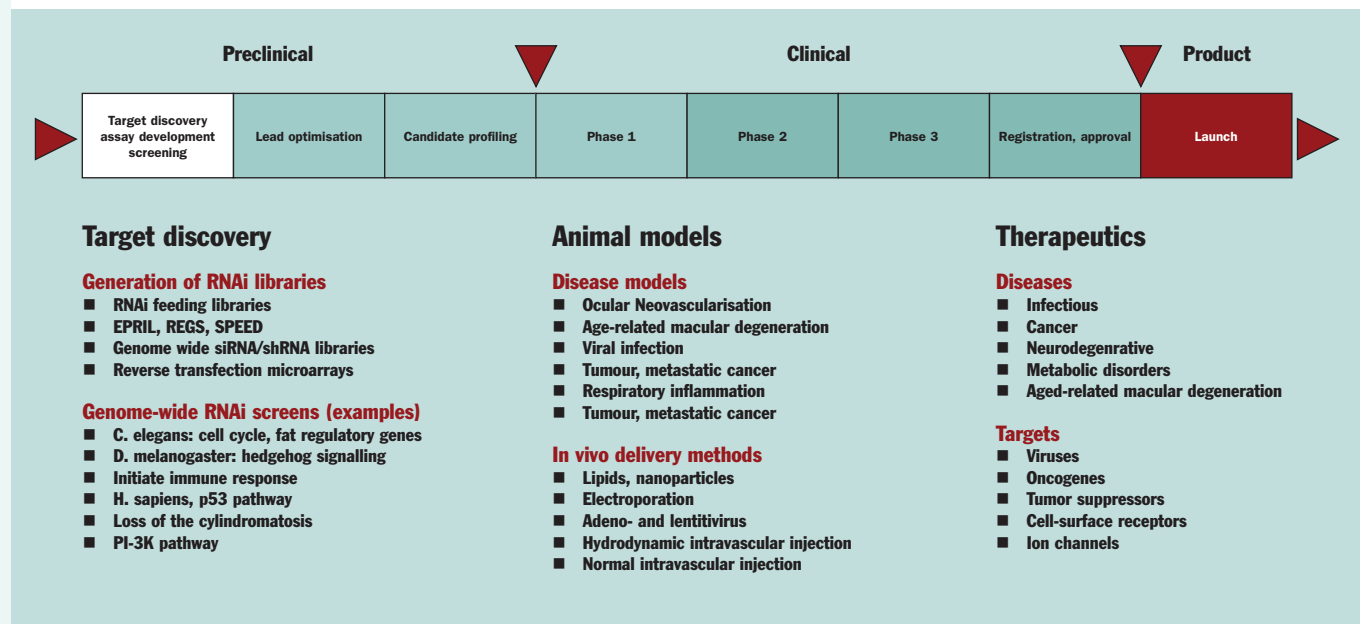
A variation of the theme generates miRNA instead of siRNA. In contrast to siRNAs that are generated by random processing of dsRNA, miRNA duplexes are excised in a sequence-defined manner from short hairpin dsRNA precursors. In contrast to siRNAs, which are fully complementary to their target mRNAs, the miRNAs bind in most cases to the 3' untranslated regions with only partial complementarity, resulting in translational arrest without cleavage of the target. Several hundred miRNAs have been found in plants and mammals, but their role has not yet been linked to particular mechanisms. This is complicated by the finding that one miRNA has several targets.

Small RNAs have diverse biological roles, such as antiviral defence, gene regulation and chromatin condensation. Since the discovery that dsRNA can inhibit expression of homologous genes, RNAi has become one of the most widely used methods for studying loss-of-function phenotypes in model organisms as well as mammalian cells, and represents the latest addition to the family of antisense technologies. RNAi is increasingly used across the whole pharmaceutical research process (see Figure 1).

RNAi in target discovery

The discovery of RNAi coincided with the explosion of genomic sequencing projects. RNAi applications can be scaled up for use in high-throughput techniques, which led to the creation of genome-wide RNAi reagents and applications. This will accelerate the drug discovery process and identification of critical pathways or the disease manifestation. Large-scale RNAi screens have already reproduced many previously hard-won genetic discoveries, and have identified new genes that appear to function in well-studied biological processes.

Figure 1. Application of RNAi along the drug development process



There are many ways to produce siRNAs, such as chemical synthesis, in vitro transcription or RNaseIII/Dicer digestion of long dsRNAs. A disadvantage of the application of such siRNAs is the transient gene expression-inhibition effect. So more effective, stable and inducible long-term gene-silencing RNAi-mediating systems have been developed. siRNAs and miRNAs can be processed from endogenously transcribed shRNA that are expressed from transfected plasmids, PCR cassettes or viral vectors. These strategies have been shown to produce functional siRNAs that silence the homologous gene in mammalian cells.

In addition to its reverse-genetic approaches, double strand-mediated gene silencing has been adapted to move genetics forward in both model organisms and mammalian cell systems to discover genes responsible for complex cellular phenotypes by the use of large-scale collections of RNAi molecules or constructs targeting all predicted genes. Combined with functional genomics applications such as transcriptional profiling and sophisticated high-content cell-based assays, this provides a unique opportunity to perform high-throughput genetics in these complex cell systems. These RNAi screens have identified new genes that function in studied biological processes.

While siRNA-mediated gene knockdown was first reported to be highly specific, recent studies demonstrated RNAi-mediated 'off-target' gene modulation resulting from mRNA cleavage or translational repression of genes with partial homology to either strand of the duplex siRNA and other toxic effects that can generate measurable phenotypes. siRNA signatures are a sum of on and off-target gene regulation. Combining high specificity with high potency is the most important challenge facing

traditional antisense oligonucleotides and siRNA. Both can affect unintended targets that are partially complementary.

Emerging use of RNAi in vivo

Mammalian tissue culture and animal models are indispensable tools to study the genetic basis of human physiology and disease. Systematic manipulation of the genetic background by overexpression, deletion or mutation of genes is the main method for understanding complex biological processes. Transgenic, knockout and knockin mice are often the best available in-vivo models for human disorders. However, generating these genetically engineered animals requires a huge amount of time, money and effort. Creating a more complex genetic environment with simultaneous gain- and loss-of-function mutations of multiple genes, as seen in human diseases, is beyond the reach of these technologies and model systems.

The success of siRNA as a laboratory tool and for target validation has led to high expectations for siRNA as an in vivo platform. However, there have been few reports of the use of siRNA in animal models, and the usefulness of synthetic siRNA in animal experimentation and preclinical drug development is yet to be established.

Chemical modifications offer one approach to modifying in vivo properties of siRNA; duplex RNA, in contrast to single strand DNA or RNA, can be stable in cell-culture media that contain low concentrations of serum, but is less stable in vivo with higher serum concentrations. Chemical modifications are developed to improve thermal stability, resistance to nuclease digestion and pharmacokinetic properties as well as to reduce non-specific

Figure 2. Therapeutic potential of RNAi

■ RNAi can be harnessed to prevent the expression of virtually any RNA target.
 ■ The possible targets for various diseases range from viral infection and oncogenes to growth factors and single nucleotide polymorphisms.

<p>HIV-1</p> <ul style="list-style-type: none"> ■ p24 HIV-1 capsid protein ■ Rev HIV-1 regulatory protein ■ Vif HIV-1 regulatory protein ■ Tat HIV-1 regulatory protein ■ HIV-1 LTR <p>Other viruses</p> <ul style="list-style-type: none"> ■ Poliovirus capsid ■ Poliovirus RNAP ■ HPV E6 mRNA HPV E7 mRNA ■ RSV P protein ■ RSV F protein Fusion 	<ul style="list-style-type: none"> ■ Hepatitis C non-structural protein 5B ■ NS5B viral polymerase mRNA injection <p>Oncogenes</p> <ul style="list-style-type: none"> ■ Ras(V12), point mutations ■ bcr-abl oncogene ■ MDR1, multidrug resistance <p>Tumour suppressors</p> <ul style="list-style-type: none"> ■ p53 tumour suppressor ■ 53bp1 	<ul style="list-style-type: none"> ■ p73Dn ■ AMD ■ VEGF-1 <p>Cell-surface receptors</p> <ul style="list-style-type: none"> ■ Fas receptor ■ CD4 Cell surface receptor ■ HIV-1 coreceptor ■ CCR5 Cell surface receptor; ■ HIV-1 coreceptor ■ CXCR4 ■ CD25 IL2 receptor α
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effects and to regulate distribution to target tissues. Optimising various routes of delivery will be necessary to maximise the potential of siRNA in animal models and pharmaceuticals.

Towards the clinic

As most diseases are fundamentally gene-based where particular proteins are overexpressed or expressed in aberrant anatomic sites, much effort has gone towards finding drugs that can downregulate gene expression on the DNA, RNA or protein level. Similar approaches are being tested to fight viruses that, upon infection, turn host cells into factories producing multiple copies of their viral genomes. One approach to alter levels of gene expression occurs on the post-transcriptional level through the use of antisense, ribozyme and RNAi-based technologies. Soon after the discovery and application of RNAi as a research tool, its potential application to therapeutics received much attention, as it may provide new therapeutics for treating infectious and neurodegenerative diseases, septic shock, macular degeneration, cancer and other illnesses. Human immunodeficiency virus, hepatitis or influenza have been successfully targeted using either siRNA or shRNA vectors.

RNAi therapeutics can be divided into two classes: topic RNAi therapeutics administers directly at sites of disease and systemically administered through the bloodstream. They are attractive therapeutic tools due to characteristics such as their potentially high degree of specificity compared with small molecules, the ability to knock down multiple genes simultaneously, and higher potency, such as lower dose compared with antisense RNAs. Therapies based upon RNAi may have some additional inherent, fundamental benefits, such as harnessing natural pathways and the potential to target virtually any protein. In light of the sequenced human genome it is theoretically possible to design siRNA for every gene, which opens the space for proteins that do not fit into the druggable target classes and allows simpler discovery of drug candidates in contrast to excessive lead optimisation steps for small molecules.

Some of the key challenges of moving RNA to the clinic are supply, stability and specificity. Once a target gene is chosen for down regulation, an effective target site on the minia or viral RNA must be chosen that does not cause off-target effects

on gene expression. One must decide whether to use saran or shrank, and whether or not it should be chemically synthesised or expressed in vivo from a range of vectors. These decisions will affect the method of delivery of the therapeutic in vivo, which remains a significant obstacle. For development of therapeutics, it is important to demonstrate that each inhibitor affects expression of only the intended gene and not unrelated genes.

With the therapeutic potential of RNA comes an interest in rapidly delivering drug candidates to the clinic. However, this faces the challenge of novel drug development, and therefore the commercialisation will take time and considerable effort.

Outlook

Since its initial discovery in 1998 by Fire et al, RNAi has become a fascinating area with enormous potential. Small non-coding RNAs can interfere with gene expression in several ways:

- By cleaving the mRNA in a sequence-specific manner
- By preventing translation of the mRNA
- By transcriptional silencing

In light of the sequenced human genome, it is theoretically possible to design siRNA for every gene.

Although the enzymology of these processes is just beginning to be understood, these RNAs are ready for application in plants and animals as the method of choice because of efficiency and ease of use enables researchers to generate deficient phenotypes without mutating the gene. The generation of additional RNAi protocols for genome-wide screens will assist with the rapid identification of genes involved in specific biological processes. siRNAs are most often used for gene functional analysis but with a therapeutic application as a logical consequence.

Despite the first animal model studies looking encouraging, developing siRNA for efficient gene silencing in vivo is challenging and many issues must be addressed before use in animals can become routine. The delivery of the RNAs to particular cells or organs is one of the tasks. As with any compound, issues of adsorption, distribution, metabolism and excretion are huge obstacles with the duplex nature of siRNA introducing a layer of complexity. Development of more efficient delivery and regulated tissue-specific or differentiation-dependent expression of siRNA/shRNA are critical issues for transgenic studies and gene therapy. The still poorly understood function of miRNA and the heterochromatin formation by methylation of DNA and histone renders itself to an application.

Though the promise of RNAi is yet to be fulfilled, its potential is beginning to be realised. In a few years RNAi will be looked upon as a 'stargate' to a new class of medicines, as recombinant DNA and antibodies have been before. **END**

