DHR International Journal Of Biomedical and Life Sciences (DHR-IJBLS)
ISSN: 2278-8301, Vol. 3(1), 2012
Available online *http://www.doublehelixresearch.com/DHRIJBLS*

Gene Silencing, Mechanism and Applications

Syed M Shah[1], N Saini [1], S Ashraf[1], G Ravi Kumar[2]
[1] Animal Biotechnology Center, National Dairy Research Institute, Karnal, India
[2] Department of Animal Biotechnology, Indian Veterinary Research Institute, Bareilly, India
Correspondence to: Syed M Shah

Abstract

Gene silencing is arguably the hottest topic in science at present and has been proclaimed to be the biggest breakthrough of the year 2002, as per the journal Science. Gene expression studies have always fascinated the scientists and are consequently studded with a number of nobel prizes. Gene silencing refers to general processes of interruption or suppression of transcription or translation of the mRNA of the target gene by mechanisms other than genetic modification. It is mediated by small RNA molecules known as siRNAs or miRNAs. These are produced by Dicer either from exogenously introduced dsRNAs or from endogenously transcribed MIR genes. These further activate RISC which inhibits transcription or translation of the target mRNA. Gene silencing maintains the genomic structure, differentiation and maintenance of stem cells and provides a promising treatment for diseases like AIDS and cancer, if the various limitations like problems of in vitro delivery and off- target effects, associated with its applications as a curing tool, are overcome.

Introduction

RNA interference (RNAi) is arguably the hottest topic in science at present. The number of papers published on RNAi has exploded over the last few years and the journal Science proclaimed it the biggest scientific breakthrough of 2002. Why is RNAi causing such a fuss? Well, imagine you could identify the role of a gene in a disease by switching it off easily, in the space of just a day, and in almost any organism. Imagine you could then take this tool and treat certain diseases, such as cancer or AIDS, by switching off the causative genes. That's the promise that RNAi offers. Gene silencing is a general term describing epigenetic processes of gene regulation. It is used to describe the "switching off" of a gene by a mechanism other than genetic modification, i.e; a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell. The interruption or suppression of the expression of a gene at transcriptional or translational level is referred to as gene silencing. The silencing of a gene could be achieved by:

i) **Drugs**: These bind to target protein and cause protein inhibition
ii) **RNase H–independent ODNs**: These oligo deoxynucleotides
iii) hybridize to target mRNA and cause inhibition of translation of target protein.
iv) **RNase H–dependent ODNs**: These hybridize to target mRNA and

mediate its degradation by RNase H.

v) **Ribozymes and DNA enzymes**: These catalyze cleavage of mRNA and hence cause its degradation.

vi) **SiRNA and miRNA**: These hybridize to target mRNA by antisense strand and guide it into endoribonuclease enzyme complex, thereby causing its degradation or inhibition of translation.

History and Discovery

1. Rich Jorgensen *et al.* in an attempt to alter flower colours in petunias, introduced additional copies of a gene encoding *chalcone synthase*, key enzyme for flower pigmentation, into flowers of normally pink or violet colour. Unexpectedly the flowers produced were less pigmented, fully or partially white. It was observed that both the transgene and endogenous gene were down regulated in white flowers. This phenomenon was called ***co-suppression of gene expression.***

2. Quelling was observed in fungus, *Neurospors crassa,* in an attempt to boost production of orange pigment produced by the gene ***aL1*** of the fungus. Attempts to enhance orange pigment in the fungus by introducing extra copies of carotenoid pigment genes failed when the orange pigment gene was suppressed in a third of

the engineered mould. In some strains, the effect was passed on through multiple generations. This was later found to be similar to post – transcriptional silencing[1].

3. Plant virologists working on improving plant resistance to viral diseases observed a similar unexpected phenomenon. It was observed that plants carrying only short, non-coding regions of viral RNA sequences would show similar levels of protection as the plants expressing virus specific proteins. It was believed that viral RNA produced by transgenes could also inhibit viral replication. The reverse experiment in which short sequences of plant genes were introduced into viruses showed that targeted genes were suppressed in an infected plant. This phenomenon was labeled **virus induced gene silencing (VIGS)** and the set of such phenomenon was called as post transcriptional gene silencing[2].

4. Guo and Kempheus, attempted to use antisense RNA to shut down expression of the ***par1*** gene in *Caenorhabditis elegans* in order to assess its function. As expected injection of antisense RNA disrupted expression of *par-1* but injection of the sense strand control also did. The result remained a puzzle for three years and for this phenomenon they coined the term **antisense mediated silencing**.

5. Three years later **Andrew Fire** and **Craig C.Mello** studied phenotypic effect of single-stranded and double- stranded ***unc-22*** RNA into gonads of *C .elegans* (1998). They observed that only the double stranded RNA consisting of both sense and antisense strand produced the typical twitcher in *C. elegans* while sense and antisense strands individually did not produced the twitcher. They concluded the results of their experiments as[3]:

i) Silencing was triggered by injecting dsRNA but weakly or not at all by ssRNA

ii) Silencing was specific for an mRNA homologous to dsRNA

iii) The dsRNA had to correspond to mature mRNA sequence, neither intron nor promoter sequence triggered the response.

iv) Targeted mRNA was degraded.

v) dsRNA are amplified in cell, as very few are required to produce the effect.

vi) Effect of dsRNA spread between tissues and even to progeny**.**

For their discovery of gene silencing by double stranded RNA they coined the term RNA

interference (RNAi) and were subsequently awarded the **Nobel prize in physiology or medicine (2006).**

6. RNAi was also observed subsequently in insects[4], frog[5] and other animals including mice[6] and in humans.

7. In 2001, Thomas Tuschl, discovered with his colleagues that RNAi could be prompted through the use of shorter pieces of RNA known as small interfering RNAs (siRNAs). Soon thereafter, they showed that duplexes of 21-nucleotide siRNAs mediated RNAi in cultured mammalian cells and demonstrated that siRNAs could be designed to silence specific genes without activating the interferon response. In other words, scientists could potentially silence any gene of interest in a highly predictable, reproducible, and accurate fashion[7].

8. Gregory Hannon and his colleagues identified, described, and named the "Dicer" enzyme, which chops dsRNA into siRNAs, as well as the RNA-induced silencing complex (RISC), which mediates the silencing process by degrading the homologous mRNA[7].

9. In 2002, the use in mammalian cells of so-called short hairpin RNAs (shRNAs), which generate endogenous siRNAs within cells and thus provide stable, heritable gene silencing (in contrast, administered siRNAs are transient in their silencing effect). This effect was named "short hairpin-activated gene silencing" or SHAGging. This discovery allowed the development of cell lines and animal models with permanently silenced genes, a major step forward for basic science in general, and especially for functional genomics[7].

Cellular components of Gene Silencing

- MicroRNAs (miRNAs)
- Small interference RNAs (siRNAs)
- Dicer
- RISC
- Histones
- Chromatin and Heterochromatin
- Transposons

Levels of Gene Silencing:

1. Post transcriptional gene silencing (PTGS)

It is known commonly as RNA interference (RNAi). It causes silencing by destruction of the mRNA of the gene to which the siRNA shows perfect complementarity.

2. Transcriptional gene silencing (TGS)

It Causes gene silencing by:

- DNA methylation.
- Heterochromatin formation.
- Programmed DNA elimination.

RNA interference (RNAi)

The term RNA interference (RNAi) was coined after the discovery that injection of dsRNA into the nematode *Caenorhabditis elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA[3]. RNAi is a cellular mechanism that degrades unwanted RNAs in the cytoplasm but not the nucleus. RNA silencing is a sequence specific RNA degradation process that is triggered by the formation of double stranded RNA that can be introduced by virus or transgene[8].

RNA interference (RNAi) is a molecular mechanism in which fragments of double stranded nucleic acid (dsRNA) interfere with the expression of a particular gene that shares a homologous sequence with the dsRNA.DsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA [9].

The dsRNA can be either, MicroRNA (miRNA) or Small interference RNA (siRNA)

Salient features of RNAi[8]

- Double stranded RNA rather than single–stranded antisense RNA is the interferring agent.

- High degree of specific gene silencing with less effort.
- Highly potent and effective, only a few double stranded RNA molecules per cell are required for effective interference.
- Silencing can be introduced in different developmental stages
- Systemic silencing
- Avoids problems with abnormalities caused by a knocked out gene in early stages which could mask desired observations.
- Silencing effects passed through generations

MicroRNA (miRNA)

These are double stranded RNA molecules which originate endogenously from ***MIR*** genes, i.e; these originate from single stranded RNA. MicroRNAs (miRNAs) constitute a novel, phylogenetically extensive family of small RNAs (~22 nucleotides) with potential roles in gene regulation. miRNAs are produced by Dicer from the precursors of ~70 nucleotides (pre-miRNAs). Some miRNA genes have been found in close conjunction, suggesting that they are expressed as single transcriptional units[10]. miRNAs which are 21–24 base duplexes that are usually incompletely based paired and form partial duplexes within the 3′-untranslated region (UTR) of targeted transcripts via an association with RISC. Their functional roles vary depending on the organism, but in mammals the primary mechanism of miRNA action is to inhibit mRNA translation[11]. These clustered miRNAs are expressed polycistronically and are processed through at least two sequential steps:

(i) generation of the ~70 nucleotide precursor microRNAs from the longer transcripts (termed pri-miRNAs);

(ii) processing of pre-miRNAs into mature miRNAs.

MicroRNA biogenesis

These are fragments originally from large DNA sequence,that has miRNA sequence and reverse complement present. Mature miRNAs are derived from two major processing events, driven by sequential cleavage by the RNAse –III enzymes Drosha and Dicer.

miRNAs are transcribed by RNA polymerase II, producing primary microRNAs (pri-miRNAs). These are several kilobases long, polyadenylated and capped, similar to the production of mRNAs from protein encoding genes. These pri-miRNAs are then subjected to processing by the microprocessor complex, composed of Drosha and its associated binding partner, Pasha (also known as DGCR8),Which results in the excision of a 65-75 nucleotide stem-loop structure called as precursor microRNA (pre-miRNA). These pre-miRNAs are then recognized and transported from the nucleus to the cytoplasm via the Ran-GTP dependent nuclear transmembrane protein, Exportin 5, where they are subjected to a second cleavage step by Dicer[12]. Processing by Dicer results in the production of a small double- stranded miRNA duplex containing 2-nucleotide- long 3′overhangs[13]. These double-stranded products are quickly unwound by helicase and a single mature strand is produced which is asymmetrically incorporated into RNA-induced silencing complex (RISC) where they can act by translational repression by a cleavage incompetent RISC or mRNA degradation by a cleavage-competent, slicer-containing RISC[14].

Small interfering RNAs (siRNAs)

These are double stranded RNA from exogenous sources like viruses, transgenes or transposons, i.e; they originate from double stranded RNAs. These result from the Dicer enzyme cutting up a larger fragment of perfectly complimentary double

stranded RNA. They have Symmetric 2nt 3'overhangs, 5' phosphate groups and are 19- 24 nucleotides in length, slightly shorter than miRNAs. These fragments then bind to the nuclease complex RISC and serve as a guide to recognition of complementary base pairing on target mRNA, ultimately bringing its degradation.

Structure of siRNA and miRNA

The final structure of both mature siRNA and miRNA is same except that miRNA is a little shorter. These are around **21-25** base pairs long and have 5' phosphates and 3' hydroxyl groups and 2 to 3 nucleotide overhangs on the 3' end. The 5'-phosphate is essential for their activity. One of their strands is known as antisense or guide strand while the other is known as passenger strand. siRNA is unwound by RISC activity and **antisense or guide strand** is left to bind to target mRNA while the **passenger strand** is degraded[15].

Mechanism of gene silencing

The mechanism of gene silencing is broadly divided into two major steps: initiation step and effector step In the initiation step the "trigger" dsRNA molecule ,usually several hundred base pairs long ,is cleaved to form 21- 23bp double-stranded fragments known as short interfering RNAs or (siRNAs) or guide RNAs. siRNAs are produced when the enzyme Dicer, a member of the RNAse III family of dsRNS – specific ribonucleases, processively cleaves dsRNA in an ATP dependent processive manner.

In the effector step the duplex siRNA are then unwound by the helicase activity associated with a distinct multiprotein complex known as RNA – induced silencing complex or RISC. An ATP dependent unwinding of siRNA duplex is required for activation of RISC. The siRNA strand that is complementary to the targeted mRNA is then used as primer by an RNA dependent RNA polymerase (RdRp) to convert the cognate mRNA into dsRNA itself. This dsRNA form of mRNA then becomes a substrate for Dicer cleavage activity, which leads to the destruction of the mRNA and formation of new siRNAs. Effectively this step amplifies the RNAi response and creates a self- perpetuating cycle of "degradative polymerase chain reaction" that will persist until no target mRNAs remain. This basic 'core' pathway defines the RNAi response as one of the most elegant and efficient biochemical mechanisms in nature. miRNAs, if they show perfect complementarity with the target mRNA cause RISC activation and target mRNA degradation similar to that by siRNA. If they do not show perfect complementarity with the target mRNA which is usually the case, they cause RISC activation similar to that by siRNAs but the activated RISC binds to the target mRNA at its 3'-end and prevent its translation, thereby inhibiting gene expression.

Transcriptional gene silencing

The siRNAs work not only at the posttranscriptional stage but also leave their indelible marks on the genomes to repress the gene transcription activity or selectively remove portions of the genomes, especially of protozoans. Broadly speaking, the siRNAs bring about three different biochemical end products with the chromatin DNA: DNA methylation, as revealed mostly in plant systems; heterochromatin formation; and programmed elimination of DNA. DNA methylation had been reckoned a major source of transcriptional gene silencing (TGS), and mechanistically TGS had been viewed very distinctively from PTGS in the past. But recent developments have caused a blurring in the identity between these two pathways. The discoveries of such epigenetic changes have ignited a revolution not only in the field of gene regulation but also in gene maintenance and gene evolution.

RNA-Dependent DNA Methylation[16]

A role for RNA in guiding *de novo* cytosine methylation of homologous DNA sequences was first discovered in viriod-infected plants and subsequently also in nonpathogenic plant systems. When the dsRNA degradation mediated PTGS occurs in plants, the genomic

DNA regions homologous to dsRNA are often found methylated at almost all the sensitive cytosine residues. This process is generally referred to as RNA-dependent-DNA methylation and the corresponding part of the genome, especially the promoter region might remain transcriptionally silent. The initiator of RNA-dependent DNA methylation / TGS could be either the transgene-derived dsRNA or the consequent siRNA. Depending on the sequence information of the dsRNA, RNA-dependent DNA methylation was found to occur at the open reading frame and/or the promoter region of the genome. If methylation occurred only at the open reading frame, TGS did not result. However, RNA-dependent DNA methylation at the promoter sequences induced TGS, which, unlike PTGS, was stable and heritable.RNA-dependent DNA methylation within the host genes has also been found to occur preponderantly during virus-induced gene silencing, a type of RNAi that is generally initiated by plant virus vectors carrying portions of host genes It is now known that the movement of transposons was controlled by transcriptional suppression (TGS) and that methylation also played a role in this suppression, depending on the nature of the transposon. The level of target DNA methylation is directly related to the amount of siRNA present in the cell, and thus the apparent differences between these observations can be resolved. In other words, the availability of siRNA may determine the level of RNA-directed DNA methylation. In the events of RNA-dependent DNA methylation, the chromodomain containing DNA methylases acts either alone or in combination with other proteins, such as piwi-containing proteins, to form complexes with the siRNAs and cause sequence-specific RNA-dependent DNA methylation, finally resulting in TGS.

RNA-dependent DNA methylation has been reported only in plants until now. Aufsatz *et al.* have also shown that asymmetric non-CpG methylation is mostly affected by RNA-dependent DNA methylation, but the existence of non-CpG methylation in mammals has always been a contentious issue. Mammalian DNA is methylated mostly at symmetric CpG or CNG sites by various forms of DNA methyltransferases. However, using a dual-labeling nearest-neighbor technique and the bisulfite genomic sequencing methods, Ramsahoye *et al.* found that the genomes of embryonic stem cells but not that of somatic tissues harbored non-CpG methylation, which accounted for 15 to 20% of total cytosine methylation. This methylation is perhaps caused by the methylase Dnmt 3a, which is highly expressed in embryonic stem cells but poorly expressed in somatic tissues. Other studies have also revealed that in *D. melanogaster* and mammals, non-CpG methylation is an early embryonic event, and this methylation can be catalyzed by Dnmt 2, which is primarily active at the initial stages of development. Hence, if RNA-dependent DNA methylation occurs at all in animals, it might be limited to the early developmental stages when the effector proteins may be found in abundance. In contrast, RNA-dependent DNA methylation is observed throughout plant development, implying the continuous availability of the appropriate plant DNA methyltransferases.

Heterochromatin Formation

Even for organisms in which RNA-dependent DNA methylation is supposedly absent, there is growing evidence that RNAi processes cause chromatin modifications leading to TGS. Generally, in eukaryotic systems, histone modifications make the chromatin structure inert to transcription by heterochromatin formation, which is modulated greatly by the

RNAi processes, as recent discoveries have revealed. In almost all organisms heterochromatin formation requires that histone H3 of the chromatin be deacetylated and then methylated at lysine 9. The SET domain of a special group of histone methyltransferases carries out this function. This methylated lysine is subsequently bound by a heterochromatin binding protein, HP1. The binding of the chromodomain containing HP1 to Met H3-K9 is highly specific and of very high affinity[25] .This binding may be followed by multimerization of HP1 and complex formation with other chromatin-remodeling proteins. As a result of this multicomplex formation, the chromatin becomes condensed and locked in a transcriptionally repressed heterochromatic state. Once formed, the heterochromatin spreads a large distance due to cooperative protein-protein interactions of chromatin-remodeling factors. In the wild-type scenario, one strand of the centromeric region is constitutively expressed, whereas the complementary strand, which is subjected to heterochromatic repression, is occasionally transcribed[26]. Such transcription will lead to the formation of dsRNA, which will be processed by the RNAi machinery. This processing might even be a nuclear step, since a component of this machinery, the RdRP, was found to be physically bound to the outer repeats of the centromeric region in a chromatin immunoprecipitation assay. The siRNA thus formed might enter a complex containing the histone methyltransferase enzyme. This complex could be a nuclear equivalent of the RISC complex (Nu.RISC) lacking nuclease activity. Such a complex would be guided to the appropriate DNA region following the DNA-RNA base pairing rules, and the histone H3-K9 of the region might be methylated to eventually generate the heterochromatin structure. Since RdRP is found locally, the spread of the heterochromatic structure may be associated with the extension of the 3' end of the siRNA primer. It has also been shown in *N. crassa* and *A. thaliana* that H3-K9 methylation directs DNA methylation. The methylated DNA could be complexed further with the methyl-binding proteins. Following these binding events, the chromatin structure will be extremely compact and condensed and would remain transcriptionally inert.

DNA Elimination

The most dramatic effect of siRNA-mediated heterochromatin formation followed by chromosomal DNA elimination and rearrangement has been recorded in the ciliated protozoan *Tetrahymena pyriformis*. Among unicellular organisms, *T. pyriformis* is unique because of its nuclear dimorphism. The two nuclei, the micronucleus and macronucleus, serve different functions. The polyploid macronucleus is the transcription center of the cell during vegetative growth, whereas the diploid and transcriptionally inert micronucleus acts as the germ line nucleus. During conjugation, the micronucleus gives rise to the macronucleus, and this transition is accompanied by two interesting and peculiar recombinant events. First, approximately 6,000 internal eliminated sequences of five pairs of micronucleus chromosomes, accounting for about 15% of genomic micro-DNA, are removed. Second, the remaining parts of these chromosomes are broken into 200 to 300 minichromosomes concomitant with the deletion of <50 nucleotide breakage eliminated sequences[16].

Mochizuki *et al.* showed that in wild-type cells of *Tetrahymena pyriformis*, siRNAs of about 26 to 31 nucleotides were produced which hybridized to micronuclear genomic DNA and not the macronuclear DNA, indicating that these

siRNAs could be internal eliminated sequence/breakage eliminated sequence-specific and are referred to as scan RNAs. The bidirectional transcription that occurs across the internal eliminated sequence repeats may form the dsRNA, which would give rise to the scan RNAs following the action of RNAi-related Dicing complexes that perhaps also include the Twi1 and PDD proteins. These scan RNAs eventually may be associated with the nuclear equivalents of RISC factor in the new macronucleus to provide heterochromatic sites at the internal eliminated sequence/breakage eliminated sequence regions. The chromodomain containing PDD proteins may remain bound to the scan RNA and thus guide to destroying the cognate DNA[28]. As an extension of this work, Yao et al. found that a similar RNAi process recognized and deleted a foreign neomycin resistance gene of bacterial origin which was integrated in a *Tetrahymena* chromosome. These two studies together strongly suggest an siRNA- (or scan-RNA)-based mechanism that controls genome-wide DNA arrangements and provides genomic surveillance against invading foreign DNAs. Thus, the *Tetrahymena pyriformis* as well as *S. pombe* data show how dramatic the epigenetic consequences of the genome could be following the formation of siRNA molecules in cells. Discovery of the link between the RNAi processes and the epigenetic chromatin modification as well as chromosome behavior is probably the most fascinating and novel face of regulation of gene silencing mechanism. The RNAi machinery is reported to control many explosive features of cellular biology, namely stem cell maintenance, cell fate determination, nonrandom chromosome segregation, etc[16].

Significance and applications

1. Biological functions

a) Immunity:

RNA interference is a vital part of the immune response to viruses and other foreign genetic material, especially in plants where it may also prevent self-propagation by transposons. It is known to be the most ancient antiviral immune response.

b) Downregulation of genes:

Endogenously expressed miRNAs, including both intronic and intergenic miRNAs, are most important in translational repression and in the regulation of development, especially on the timing of morphogenesis and the maintenance of undifferentiated or incompletely differentiated cell types such as stem cells

c) Upregulation of genes

RNA sequences (siRNA and miRNA) that are complementary to parts of a promoter can increase gene transcription, a phenomenon dubbed RNA activation. Part of the mechanism for how these RNA upregulate genes is known: dicer and argonaute are involved, and there is histone demethylation

d) Evolution:

Based on parsimony-based phylogenetic analysis, the most recent common ancestor of all eukaryotes most likely already possessed an early RNA interference pathway; the absence of the pathway in certain eukaryotes like *Leishmania major, T. cruzi, S. cervisae and* certain *ascomycetes* & *basidiomycetes* is thought to be a derived characteristic. This ancestral RNAi system probably contained at least one dicer-like protein, one argonaute, one PIWI protein, and an RNA-dependent RNA polymerase that may have also played other cellular roles. A large-scale comparative genomics study likewise indicates that the eukaryotic crown group already possessed these components, which may then have had closer functional associations with generalized RNA degradation systems such as the exosome. This study also suggests that the RNA-binding

argonaute protein family, which is shared among eukaryotes, most archaea, and at least some bacteria (such as *Aquifex aeolicus*), is homologous to and originally evolved from components of the translation initiation system. The ancestral function of the RNAi system is generally agreed to have been immune defense against exogenous genetic elements such as transposons and viral genomes.Related functions such as histone modification may have already been present in the ancestor of modern eukaryotes, although other functions such as regulation of development by miRNA are thought to have evolved later[29].

RNA interference genes, as components of the antiviral innate immune system in many eukaryotes, are involved in an evolutionary arms race with viral genes. Some viruses have evolved mechanisms for suppressing the RNAi response in their host cells, an effect that has been noted particularly for plant viruses. Studies of evolutionary rates in *Drosophila* have shown that genes in the RNAi pathway are subject to strong directional selection and are among the fastest-evolving genes in the *Drosophila* genome[30].

2. Technological applications

a) Gene knockdown:

The RNA interference pathway is often exploited in experimental biology to study the function of genes in cell culture and in vivo in model organisms. Double-stranded RNA is synthesized with a sequence complementary to a gene of interest and introduced into a cell or organism, where it is recognized as exogenous genetic material and activates the RNAi pathway. Using this mechanism, researchers can cause a drastic decrease in the expression of a targeted gene. Studying the effects of this decrease can show the physiological role of the gene product. Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as a "knockdown", to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated. Extensive efforts in computational biology have been directed toward the design of successful dsRNA reagents that maximize gene knockdown but minimize "off-target" effects.

Specialized laboratory techniques have also been developed to improve the utility of RNAi in mammalian systems by avoiding the direct introduction of siRNA, for example, by stable transfection with a plasmid encoding the appropriate sequence from which siRNAs can be transcribed, or by more elaborate lentiviral vector systems allowing the inducible activation or deactivation of transcription, known as *conditional RNAi.*

b) Functional genomics:

Most functional genomics applications of RNAi in animals have used C. elegans and Drosophila, as these are the common model organisms in which RNAi is most effective. C. elegans is particularly useful for RNAi research for two reasons: firstly, the effects of the gene silencing are generally heritable, and secondly because delivery of the dsRNA is extremely simple, delivery by feeding.

Approaches to the design of genome-wide RNAi libraries can require more sophistication than the design of a single siRNA for a defined set of experimental conditions. Artificial neural networks are frequently used to design siRNA libraries and to predict their likely efficiency at gene knockdown. Mass genomic screening is widely seen as a promising method for genome annotation and has triggered the development of high-throughput screening methods based on microarrays. Functional genomics using RNAi is a particularly attractive technique for genomic mapping and annotation in plants because many plants are polyploid, which

presents substantial challenges for more traditional genetic engineering methods. For example, RNAi has been successfully used for functional genomics studies in bread wheat (which is hexaploid) as well as more common plant model systems Arabidopsis and maize.

c) Medicine:

It is possible to exploit RNA interference in therapy. Although it is difficult to introduce long dsRNA strands into mammalian cells due to the interferon response, the use of short interfering RNA mimics has been more successful. Among the first applications to reach clinical trials were in the treatment of macular degeneration and respiratory syncytial virus. RNAi has also been shown to be effective in the reversal of induced liver failure in mouse models. RNA interference is also often seen as a promising way to treat cancer by silencing genes differentially upregulated in tumor cells or genes involved in cell division.

AIDS: It has been shown that siRNAs can inhibit HIV replication effectively in culture. HIV infection can also be blocked by targeting either viral genes (for example, gag, rev, tat and env) or human genes (for example, CD4, the principal receptor for HIV) that are involved in the HIV life cycle. Thus promising antiviral therapies that can attack multiple viral and cellular targets circumvent genetic resistance of HIV. But these results have been achieved by transfecting the siRNAs into cells, and getting the siRNAs to function in vivo is likely to be a more difficult task. To get around the delivery problem, many groups have designed promoter systems that can express functional siRNAs when transfected into human cells.

Hepatitis: This has provided the first tangible evidence for RNAi as a therapy for diseases in live animals. Early RNAi studies noted that RNA silencing was prominent in the liver, which made this organ an attractive target for therapeutic approaches. Many immune-related liver diseases are characterized by apoptosis, which is mediated by a protein called Fas. So Judy Lieberman's group injected siRNA targeting Fas intravenously into two models of autoimmune hepatitis in mice. This decreased Fas mRNA and protein levels in hepatocytes and protected the cells against liver injury from apoptosis, even when siRNA was administered after the induction of injury. Extending these findings to other liver diseases looks hopeful, but it was concluded that other strategies, such as viral vectors, might be required to target organs in which RNA silencing is less effective than in the liver.

Cancer: Gregory Hannon and colleagues have used RNAi to silence expression of p53 — the 'guardian of the genome', which protects against any tumour-associated DNA damage — by introducing several p53-targeting shRNAs into stem cells and looking at the effect in mice. The shRNAs produced a wide range of clinical effects, ranging from benign to malignant tumours, the severity and type of which correlated with the extent to which the shRNA had silenced p53. As tumour suppressors such as p53 usually work as part of a complex and finely regulated network, the ability to dampen these networks to varying degrees in these libraries, termed as epi-allelic series of hypomorphic mutations, will be of enormous value when it comes to investigating the early stages of disease.The success of these modified stem cells also gives hope that this could treat diseases in which stem cells can be modified ex vivo and then re-introduced into the affected individual.

d) Biotechnology

RNA interference has been used for applications in biotechnology, particularly in the engineering of food plants that produce lower levels of natural plant toxins. Such techniques take

advantage of the stable and heritable RNAi phenotype in plant stocks. For example, cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid product gossypol, making them unsuitable for human consumption. RNAi has been used to produce cotton stocks whose seeds contain reduced levels of *delta cadinene synthase*, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where gossypol is important in preventing damage from plant pests. Similar efforts have been directed toward the reduction of the cyanogenic natural product linamarin in cassava plants. Previous commercial products, including the Flavr Savr tomato and two cultivars of ringspot-resistant papaya, were originally developed using antisense technology but likely exploited the RNAi pathway. Japanese researchers reported in the 19 June 2003 issue of Nature the successful construction of transgenic coffee plants that are naturally decaffeinated by knocking down a gene involved in caffeine biosynthesis.

Challenges to overcome

Things in biology have a way of becoming more complicated than anyone can foresee, and RNAi is in no way an exception. Application of RNAi for biological and clinical uses poses a number of serious challenges like in vivo delivery of the dsRNA, off-target effects, and
competition with cellular RNAi components.

Delivery, i.e; getting those exquisitely specific siRNAs or shRNAs to the appropriate sites in the appropriate amounts to ensure appropriate uptake and the intended silencing remains a considerable challenge.Experts in the field agree that delivery is a daunting barrier to successful RNAi therapy. However, RNAi biotech companies and their backers are banking on overcoming the delivery barrier, and academic researchers seem confident that it can be done. There are basically two strategies for delivering siRNAs in vivo. One strategy is gene therapy, which uses a viral vector to deliver the siRNA to the cells of interest. The other route is the chemical synthesis of the reagent, using some chemical modifications that change the properties of the siRNA such that they are more stable and are retained longer in the bloodstream; this simultaneously changes their uptake properties and allows more opportunity for uptake. With the broad applicability of RNAi to a diverse range of human diseases in a wide variety of organ systems, both delivery methods are being pursued for specific therapeutic targets. As RNAi therapies make their way into the clinic,it is perhaps inevitable that a more traditional dichotomy will emerge in delivery: local versus systemic administration. Some organs are simply much easier than others to reach with drugs. The delivery hurdles are going to be more significant for systemic uses of RNAi ,in other words, administration of siRNAs either intravenously or subcutaneously as compared to direct RNAi approach, which is the application of siRNAs to certain anatomical sites, for example the eye or the central nervous system. With direct RNAi, drugs can perform their actions at such sites without having to negotiate the gastrointestinal tract or other hurdles that must be faced to reach less accessible organs. The eye has been one of the first targets of siRNA therapeutics in development. Local delivery of an siRNA to the eye via intravitreal injection or topical administration is aimed at controlling the proliferation of abnormal blood vessels associated with one form of age-related macular degeneration. Several RNAi biotech companies are working on siRNAs designed to block the vascular endothelial growth factor pathway, a validated target of therapy in this

disease. With the liver being another relatively easy drug delivery site, other companies are also in hot pursuit of an siRNA candidate compound to treat hepatitis C. In terms of systemic delivery of RNAi therapeutic agents, some researchers believe DNA-based vectors will be the way to go. Australia's Benitec, for example, has developed a technique it has dubbed "DNA-directed RNAi" which it claims allows for the inducible transient or permanent silencing of multiple genes. That approach could prove beneficial in the treatment of diseases such as HIV/AIDS and cancer, in which combination therapy attacking multiple targets simultaneously is an accepted therapeutic stratagem[7].

'Off-target effects' compromise the specificity of RNAi if sequence identity between siRNA and random mRNA transcripts causes RNAi to knockdown expression of non-targeted genes.

The chance for RNAi off-target effects proved considerable, ranging from 5 to 80% for each of the organisms, when using as parameter the exact identity between any possible siRNA sequences (arbitrary length ranging from 17 to 28 nt) derived from a dsRNA (range 100–400 nt) representing the coding sequences of target genes and all other siRNAs within the genome. High-sequence specificity and low probability for off-target reactivity were optimally balanced for siRNA of 21nt, the length observed mostly in vivo. The chance for off-target RNAi increased (although not always significantly with greater length of the initial dsRNA sequence, inclusion into the analysis of available untranslated region sequences and allowing for mismatches between siRNA and target sequences. siRNA sequences from within 100 nt of the 50 termini of coding sequences had low chances for off-target reactivity.

It has been found that siRNAs and shRNAs can induce subsets of genes involved in the interferon response in mammalian cells. Sledz *et al.*(2003) showed that 21-bp siRNAs activate the Jak–Stat pathway and induce a global up-regulation of IFN-stimulated genes, mediated by the dsRNA-dependent protein kinase, PKR. However, their findings also confirmed that siRNA-induced knockdown is independent of the IFN system. Another report by Bridge *et al*.(2003) linked the activation of interferon to the expression vectors carrying shRNA hairpins, while siRNA alone did not elicit such response. In a complementary study, Heidel et al. (2004) showed that it was possible to administer naked synthetic DNA to mice and down-regulate an endogenous or exogenous target without inducing the IFN response. In addition to the IFN response, it has been reported that si/shRNAs initiated immune activation in macrophages and dendritic cells through toll-like receptor 3 (toll receptors recognize exogenous nucleic acids)[31] In mammalian cells it has been observed that long dsRNA molecules activate a protein kinase, PKR; activated PKR, inturn phosphorylates and inactivates the translation initiation factor,eIf2a, leading to repression of translation. Long dsRNAs activate RNAse L which inturn leads to non- specific degradation of mRNA molecules in the cell causing global inhibition of gene expression.

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