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Review

RNAi: ancient mechanism with a promising future

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Abstract

RNA interference (RNAi) is a gene silencing mechanism that has been conserved in evolution from yeast to man. Double stranded RNA, which is either expressed by cellular genes for small non-coding RNAs, by parasitic nucleic acids, such as viruses or transposons, or is expressed as an experimental tool, becomes processed into small RNAs, which induce gene silencing by a variety of different means. RNAi-induced gene silencing controls gene expression at all levels, including transcription, mRNA stability and translation. We are only beginning to understand the physiological roles of the RNAi pathway and the function of the many small non-coding RNA species, which are found in eukaryotic genomes. Here we review the known functions of genes in RNAi in various species, the experimental use and design of small RNAs as a genetic tool to dissect the function of mammalian genes and their potential as therapeutic agents to modulate gene expression in patients.

Keywords: RNA induced silencing complex; Double stranded RNA; Short interfering RNA; siRNA; shRNA; RNA interference; gene silencing

1. Introduction

Since its formal discovery in 1998 (Fire et al., 1998), RNA interference (RNAi) has rapidly developed into one of the most widely applied technologies in molecular and cellular research. RNA-induced gene silencing was first identified in the analysis of transgenic plants, in which transgenes were found to induce silencing of the homologous endogenous genes (Napoli et al., 1990; van der Krol et al., 1990). This mechanism was initially called co-suppression to indicate that a transgene could not only suppress the expression of the homologous endogenous gene but also silence itself. Co-suppression was later defined to happen at the transcriptional as well as posttranscriptional level (Matzke et al., 2001). RNA-directed transcriptional silencing involves histone H3 methylation and formation of heterochromatin (Stevenson and Jarvis, 2003), while post-transcriptional gene silencing (PTGS) is based on mRNA degradation. Hamilton and Baulcombe (1999) could show that a small 25 nucleotide (nt) RNA species derived from the target mRNA sequence was involved in the latter process.

The involvement of double stranded RNA (dsRNA) in gene silencing phenomena, however, was discovered by Fire et al., who found that dsRNA, but not single stranded sense or antisense RNA, mediated gene silencing in microinjected *Caenorhabditis elegans* (Fire et al., 1998). RNAi in *C. elegans* operates at the posttranscriptional level by increasing mRNA turnover (Montgomery et al., 1998).

A major breakthrough in the elucidation of the underlying mechanism was the biochemical analysis of RNAi using *Drosophila* embryo or cell extracts (Hammond et al., 2000; Tuschl et al., 1999; Zamore et al., 2000), which led to the identification of the dsRNA processing enzyme Dicer (Bernstein et al., 2001a) as well as the RNA induced silencing complex, RISC (Hammond et al., 2000), which executes RNAi by using the small dsRNA species generated by Dicer as guidance molecules to target the homologous, endogenous mRNA for degradation (Elbashir et al., 2001b,c; Zamore et al., 2000).

These discoveries led to the rapid improvement of RNAi tools, tailored to the needs of the various experimental systems, and triggered intense genetic and biochemical

Abbreviations: RNAi, RNA interference; RISC, RNA induced silencing complex; RDRP, RNA dependent RNA polymerase; dsRNA, double stranded RNA; shRNA, short hairpin RNA; PTGS, posttranscriptional gene silencing; siRNA, short interfering RNA; miRNA, micro RNA; stRNA, short temporal RNA.

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research into the molecular basis and regulation of RNAi (Hammond et al., 2001b; Tijsterman et al., 2002). It became clear that RNAi is a highly conserved mechanism that functions in many different cellular pathways from regulating gene expression to fighting infection and the dangers of mobile genetic elements.

Here, we briefly review the mechanism and regulation of the RNAi pathway, discuss the physiological functions of RNAi and recapitulate the various strategies to induce RNAi in mammalian cells.

2. The RNAi mechanism

The genetic and biochemical analysis of RNAi has led to a model, in which RNAi can be divided into two distinct phases: an initiation and an execution phase. The initiation phase involves processing of dsRNA into small RNA molecules, called small interfering RNAs (siRNA). In the execution phase, siRNAs are then incorporated into large ribonucleoprotein complexes. These effector complexes interfere with gene expression by using the small RNA strand to identify their complementary mRNA, which becomes cleaved and degraded. In a related pathway, short non-coding single stranded RNAs, which are derived from partially complementary dsRNA precursor molecules, are used to regulate the translation of mRNAs harbouring complementary sequences in their 3′ UTRs. Figs. 1 and 2 summarise the most important aspects of RNAi, which are described in more detail below. In addition, Table 1 provides an overview over genes known to be involved in RNAi in various species.

2.1. The initiation phase: dsRNA processing into siRNAs

siRNAs are generated from stretches of double stranded RNA (Hammond et al., 2000; Tuschl et al., 1999; Zamore et al., 2000) by Dicer, a conserved member of the RNase III gene family (Bernstein et al., 2001a). Sources of the dsRNA molecules include experimentally expressed dsRNAs, aberrantly expressed transgenes, RNA viruses, transposons, or short endogenous hairpin RNAs (Hannon, 2002). Dicer contains a C-terminal dsRNA binding domain, an N-terminal RNA helicase as well as two RNaseIII-like domains (Bernstein et al., 2001b). As single domain bacterial RNAse III cleaves dsRNA at 11 nt intervals, the presence of two structural but only one functional RNaseIII-like domains in Dicer could explain the generation of 21–23 nt long siRNA molecules with overhanging 3′ ends (Blaszczyk et al., 2001). Dicer has been identified in many organisms including fission yeast (Volpe et al., 2002), plants (Golden et al., 2002; Park et al., 2002), *C. elegans* (Knight and Bass, 2001), *Drosophila* (Bernstein et al., 2001a), mice (Bernstein et al., 2003) and humans (Provost et al., 2002; Zhang et al., 2002), suggesting that all organisms use the same basic mechanism to initiate the RNAi pathway. Interestingly, neither Dicer nor any other of the conserved proteins involved in RNAi seem to have homologues in *S. cerevisiae* (Aravind et al., 2000) or in *Archaea* and *Eubacteria*.

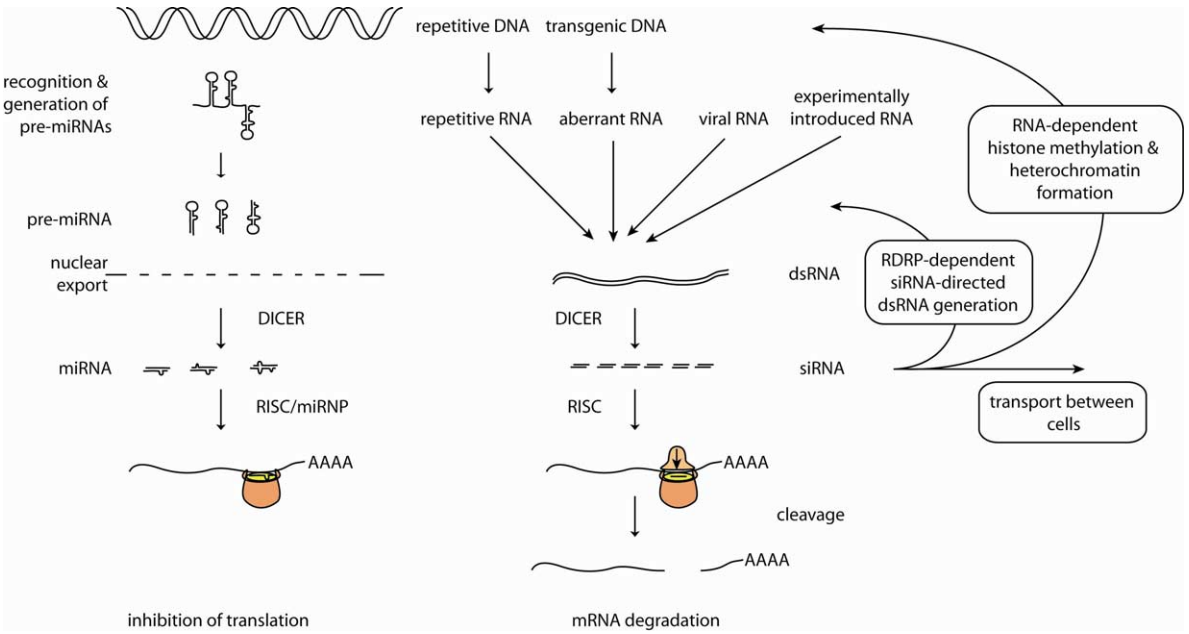

Fig. 1. dsRNA-directed gene silencing mechanisms. Short dsRNA molecules can either be expressed by endogenous genes, invading viruses or by experimental means and are funnelled into one of two different silencing mechanisms. The miRNA-dependent pathway, which mainly controls the translation of mRNAs, involves imperfect base pairing of the miRNA to its mRNA target, while siRNAs are perfectly complementary to their cognate mRNA species and induce their endonucleolytic cleavage and degradation. Although human miRNAs have been identified, their biological function in humans is currently unknown. Amplification of the RNAi signal by RDRP-dependent mechanisms, RNA-induced epigenetic control of gene expression as well as RNAi transfer between cells have been observed in some but not all species.

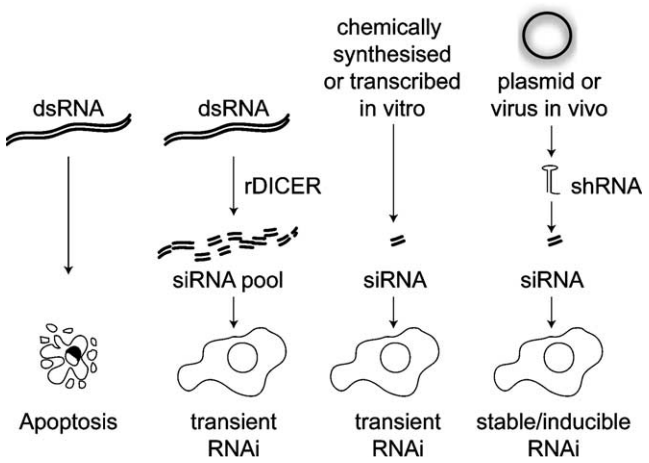

Fig. 2. Strategies to experimentally induce RNAi in human somatic cells. While long dsRNA induces a strong interferon response with consecutive apoptosis in human somatic cells, expression of short siRNAs will induce gene-specific gene silencing. siRNAs can be generated in vitro by digestion of long dsRNA into siRNAs using recombinant human Dicer or *E. coli* RNase III. Transfection of synthetic siRNAs of defined sequence is more specific than using siRNA pools, which might affect other genes as well. Stable and conditional RNAi is achieved by in vivo transcription of short hairpin RNAs, which become intracellularly processed into siRNAs by Dicer. shRNA expression cassettes can be stably integrated into the genome of human tissue culture cells and can be rendered inducible by using controllable promoters.

2.2. The execution phase: assembly of siRNA containing silencing complexes

Dicer-generated siRNAs are then incorporated into a large multiprotein complex, which is involved in various gene-silencing modes, and is called the RNA induced silencing complex, or RISC (Hammond et al., 2000; Nykanen et al., 2001). Processing of dsRNA and assembly of a functional RISC likely occurs in the cytoplasm, as Dicer is a cytosolic enzyme and RISC activity can be purified from cytosol (Billy et al., 2001). *R2D2*, a *Drosophila* gene related to the *C. elegans* RNAi gene *RDE-4*, has been implicated in the transfer of siRNAs into the RISC (Liu et al., 2003). Generation of siRNAs from dsRNA in *Drosophila* embryo extracts, unwinding of the siRNA duplex, and incorporation into the RISC require ATP (Nykanen et al., 2001). In contrast, human Dicer does not seem to require ATP for processing of dsRNA into siRNA molecules (Zhang et al., 2002).

After unwinding of the siRNA duplex, a single RNA strand becomes incorporated into a RISC complex (Martinez et al., 2002a). In the siRNA-mediated mRNA degradation pathway, the antisense strand of the siRNA molecule is used to target the cognate mRNA for degradation (Schwarz et al., 2002). This process involves specific base pairing between the antisense strand of the siRNA and the target mRNA, endonucleolytic cleavage of the mRNA strand across the middle of the siRNA strand (Elbashir et al., 2001b; Martinez et al., 2002a) and subsequent degradation of the now unprotected mRNA.

Only a few components of the RISC have been identified, but members of the Argonaute protein family feature prominently in all complexes analysed thus far. Argonaute proteins, named after the peculiar appearance of *Arabidopsis ago*1 mutants reminiscent of the homonymous squid, are conserved from yeast to humans, and are involved in many different cellular functions (Carmell et al., 2002). Eight genes encoding Argonaute proteins, which are also called PAZ-Piwi-domain (PPD) proteins, have been identified in the human genome (Sasaki et al., 2003). They are characterized by a PAZ domain, named after the *Drosophila* protein Piwi and the plant proteins ARGONAUTE1 and ZWILLE, and a C-terminal Piwi domain (Cerutti et al., 2000). The functions of these domains are unknown, but may serve in protein-protein interactions as many proteins involved in gene silencing do contain these motifs, e.g. Dicer, which contains a PAZ domain (Bernstein et al., 2001b). Several members of the Argonaute family have been identified in genetic screens designed to dissect the RNAi pathway, e.g. in *C. elegans* (RDE-1 (Parrish and Fire, 2001)), *Arabidopsis* (AGO1 (Fagard et al., 2000)) and *Neurospora crassa* (QDE-2 (Catalanotto et al., 2000)).

Other proteins of the RISC complex include fragile X mental retardation protein (FMRP) and the Vasa intronic gene, VIG (Caudy et al., 2002). These proteins have RNA-binding properties and complex with Tudor-SN, a protein related to micrococcal nucleases (Caudy et al., 2003). Whether Tudor-SN is responsible for the endonucleolytic activity of the RISC complex, or is an associated nuclease that degrades the cleaved mRNA remains to be established, because Zamore and coworkers have recently identified a different, Mg^{2+}-dependent endonuclease activity in purified RISC complexes, which cleaves single stranded RNA to leave a 5′ phosphorylated 3′ cleavage product (Schwarz et al., 2004).

2.3. miRNA-controlled gene expression

The RISC complex, or RISC-like complexes, can not only associate with siRNAs to engage in the degradation of homologous mRNA molecules, but can also associate with another species of small RNAs, called micro RNAs or miRNAs. In contrast to siRNAs, miRNAs predominate as single stranded RNA species and regulate the translation rather than the stability of their complementary mRNA target (reviewed in Carrington and Ambros, 2003). MicroRNA containing ribonucleoproteins (miRNPs) have been purified from human cells and found to contain the RNA helicase domain protein Gemin-3, Gemin-4, a protein with unknown function, as well as eIF2C, the human homologue of ARGONAUTE1 (Mourelatos et al., 2002). The Argonaute proteins eIF2C1 and eIF2C2 have also been identified in purified RISC complexes from HeLa cells

Table 1
Genes in RNAi

Name (species)	Structure and function
Initiation step	
Dicer: human Dicer, DCR-1 (Ce), Dicer-1 and 2 (Dm), DCL1 (At)	dsRNA-binding, 2 RNaseIII-; ATP-binding-, PAZ-; DEAD/DEAH box helicase domain; dsRNA processing into siRNAs; pre-miRNA processing into miRNAs. Essential in all organisms studied. DCL1 is not essential for PTGS in plants.
Drosha (Dm)	2 RNaseIII domains, dsRNA binding domain; involved in generation of pre-miRNAs
R2D2 (Dm)	dsRNA binding motif; transfer of siRNAs into RISC complexes
RDE-4 (Ce)	dsRNA binding motif, RNA helicase
RDRPs: SAD1 (Nc), QDE-1 (Nc), EGO-1 (Ce), RRF-1 (Ce), RrpA (Dd), Spn-E (Dm), SGS2 (At), SDE1 (At)	RNA-dependent RNA helicases use siRNAs to prime dsRNA synthesis. Secondary siRNAs are produced by Dicer. Putative amplification mechanisms required for systemic and heritable RNAi. No homologues identified in humans.
Execution step: components of RISC and miRNP	
Argonaute proteins:	PAZ and C-terminal Piwi domains; function unknown; protein-protein interaction
eIF2C-related: eIF2C1,2,3 (Hs); Ago1-5 (Mm), ALG-1 (Ce), AGO1 (At), ZWILLE (At)	Human eIF2C proteins are found in miRNPs; AGO1 and ZWILLE are developmental regulators
QDE-2 (Nc), RDE-1 (Ce)	Essential for RNAi in *Neurospora crassa* and *C. elegans*, resp.
Piwi-related: Piwi (Dm), Aubergine (Dm), HIWI (Hs), Miwi (Mm)	Piwi: nuclear protein, regulates germline stem cell fate in *Drosophila* Aubergine: cytoplasmic, required for RNAi in *Drosophila* oocytes
Others:	
Gemin 3 (Hs)	RNA helicase domain; complexes with eIF2C2 and Gemin 4
Gemin 4 (Hs)	Function unknown
FMRP (Hs, Dm)	Human FMRP: RNA binding protein involved in translational control; regulates axon guidance and neuronal plasticity; mutated in patients suffering from fragile X-syndrome
VIG (Hs, Dm)	RNA binding domain
Tudor-SN (Dm)	Nuclease found in FMRP and VIG containing RISC complexes
Systemic and inheritable RNAi	
MUT-7 (Ce)	RecQ helicase domain, related to Blooms' and Werner's syndrome helicases.
RDE-2 (Ce)	Function unknown
SID-1,2,3, (Ce)	SID-1: transmembrane protein, which facilitates dsRNA uptake; homologues in humans

At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm: *Mus musculus*; Nc, *Neurospora crassa*.

(Martinez et al., 2002a), and are essential for RNAi in human cells (Doi et al., 2003).

miRNAs can be considered as regulatory RNA molecules that are encoded in mono- and polycistronic messages. These RNA precursors, which are probably transcribed by RNA polymerase II, are processed in the nucleus into pre-miRNAs. In *Drosophila*, an RNase III-related activity (Drosha) has been identified, which is involved in the generation of pre-miRNAs (Lee et al., 2003). These 60–70 nt long pre-miRNAs form stem-loop structures, or hairpins. Because the stem structures of miRNAs are not perfectly complementary to each other, pre-miRNAs are characterised by the presence of mismatches, bulges and loops in their stems (Pasquinelli, 2002). These non-coding pre-miRNAs are then exported into the cytoplasm to be further processed by Dicer (Lee et al., 2002). Matured miRNAs are of similar size to siRNAs (20–25 nt), but single stranded and recognize sequences in the 3′ UTR of their target mRNAs. Depending on the extent of homology to their target sequence, miRNAs can initiate mRNA degradation or block translation by a poorly defined mechanism (Doench et al., 2003). The molecular compositions of RISCs/miRNPs involved in mRNA degradation or inhibition of translation are still unclear, and throughout this review we use the term RNAi to include all RNA-directed gene silencing mechanisms, which involve dsRNA processing.

2.4. Transitive and systemic RNAi

In addition to the basic mechanism of RNAi, which seems to be conserved throughout evolution, RNAi has been

adapted to serve species specific functions. Dramatic examples are transitive and systemic RNAi. In transitive RNAi, dsRNA-derived siRNAs act as primers for a RNA-dependent RNA polymerase (RDRP) to amplify dsRNA, which is then cleaved by Dicer into siRNAs (Sijen et al., 2001). In *C. elegans*, transitive RNAi has strict polarity and 'transits' only 5′ from the sequence complementary to the input dsRNA (Sijen et al., 2001). In plants, transitive RNAi requires ongoing transcription and copying of the entire mRNA strand, as secondary siRNAs from sequences 5′ as well as 3′ from the primary siRNAs have been found (Vaistij et al., 2002). RDRPs possibly involved in transitive RNAi have been identified in *C. elegans* (EGO-1 (Smardon et al., 2000) and RRF-1 (Sijen et al., 2001)), filamentous fungi (QDE-1 (Cogoni and Macino, 1999a)), plants (SDE1 (Dalmay et al., 2000)) and *Dictyostelium discoideum* (RrpA (Martens et al., 2002)), but not in the genomes of *Drosophila* or humans.

Systemic RNAi, i.e. the spreading of a gene-silencing effect between cells and even throughout an organism, is another interesting feature of RNAi, which has been documented in plants and worms but, until now, not in *Drosophila* and mammals. Systemic RNAi requires an amplification mechanism to generate the RNAi signal (see above), and a means to transport the mobile silencing signal between cells (Mlotshwa et al., 2002). Systemic RNAi can most readily be demonstrated by the fact that feeding worms on dsRNA expressing bacteria can induce gene-specific RNAi throughout the animal (Timmons and Fire, 1998). Apparently, dsRNA is taken up in the gut and processed into a mobile silencing signal, possibly siRNAs, which are then transported throughout the organism to induce efficient gene silencing. RNAi can even be passed onto the progeny to cause gene-specific loss-of-function phenotypes in the F1 generation (Timmons et al., 2001). In a genetic screen to identify genes involved in systemic RNAi, three genes (*sid*-1, 2, and 3) have been identified in *C. elegans* (Winston et al., 2002). *SID-1* has been characterised in more detail and was found to encode a transmembrane protein, which can facilitate the uptake of dsRNA (Feinberg and Hunter, 2003). As homologues of *SID-1* have been identified in the genomes of humans and mice, it will be interesting to learn whether these proteins may be involved in the uptake of dsRNA. Whether or not transitive and systemic RNAi operates in human cells has broad implications for using RNAi as a therapeutic option (see below). Regulated uptake of dsRNA could explain cell type specific differences in the capacity to initiate RNAi. In addition, other mechanisms have been identified that regulate RNAi by recognising and blocking siRNAs (Kennedy et al., 2004; Vargason et al., 2003).

3. Known biological functions of the RNAi pathway

As outlined above, RNAi has been discovered by carefully conducted experiments aimed to overexpress genes in plants, or suppress the function of genes in *C. elegans* by microinjection of antisense RNA. In the first experimental setting, the outcome was exactly the opposite to the expectations, because instead of deepening the purple colour of petunias, introduction of the chalcone synthase gene resulted in loss of pigmentation (Napoli et al., 1990). Transgene-induced gene silencing has also been reported in mammalian cells but is less well characterised (Bahramian and Zarbl, 1999). In the second set of experiments to suppress the expression of genes in worms, microinjection of in vitro transcribed antisense strand RNA had the same effect as using the sense RNA strand (Guo and Kemphues, 1995). As dsRNA processing appeared to be involved in these silencing effects, genetic screens were initiated to study the molecular make-up and regulation of RNAi.

These studies revealed that RNAi-based mechanisms are used by various species in a sophisticated manner to achieve a plethora of gene-regulatory mechanisms, ranging from epigenetic control of gene activity, to antiviral defence and control of development. Given the versatility of these RNA-directed regulatory mechanisms, which will be described below, it is likely that they play important physiological functions in humans as well. Whether defects in the RNAi pathway contribute to alterations in gene expression and diseases is currently unknown. In the following, we will review recent findings on the functions of genes involved in RNAi to demonstrate how RNA-directed gene regulatory pathways have been established in different species.

3.1. RNAi and the control of mobile genetic elements

In *C. elegans*, screening for surviving mutants fed on bacteria expressing dsRNA targeting an essential developmental gene, identified genes involved in RNAi. Four complementation groups (RNAi deficient, *rde*-1,2,4) were identified. The Argonaute protein RDE-1 (Tabara et al., 1999) and the RNA helicase RDE-4 have been found to interact with Dicer (Tabara et al., 2002) and are thus likely to act in the initiation step, whereas RDE-2, whose function is still unknown, acts in the effector pathway (Dernburg et al., 2000).

Many of the above genes have also been identified in a genetic screen to illuminate the mechanism that controls the activity of mobile genetic elements, such as transposons, in the germline of the nematode *C. elegans*. Phenotypically, high transposon activity results in a 'mutator' phenotype and genes have, therefore, been named '*mut*' (Ketting et al., 1999). Several of the *mut* genes identified were allelic to genes identified in the RNAi deficiency (rde) screen. *MUT*-7 was found to be essential, together with *RDE*-2, for the effector phase and inheritance of RNAi (Grishok et al., 2000). These results implicate RNAi in the control of mobile genetic elements in the germline of *C. elegans*.

A similar function has been ascribed to the *Drosophila* RNA helicase Spindle-E/Homeless. Mutation in this gene

resulted in a loss of gene silencing, and an enhanced activity of retrotransposons, i.e. mobile genetic elements, which replicate via an RNA intermediate, in the germline (Aravin et al., 2001).

3.2. RNAi as an antiviral mechanism

Genetic screens in *Arabidopsis* aimed to identify genes mediating the silencing effect of transgenes have uncovered three complementation groups of 'suppressors of gene silencing' (*sgs*) (Elmayan et al., 1998), and three complementation groups of 'silencing defective' (*sde*) mutants (Dalmay et al., 2000). While the function of many of the identified genes is still unclear, they are believed to function in the initiation step of the RNAi pathway, e.g. *SGS2* and *SDE1* are allelic and encode an RDRP. Interestingly, mutants in some of these genes, e.g. *SGS2* and *3*, exhibit high sensitivity towards viral infection (Mourrain et al., 2000) suggesting that plant RNAi has an important function in viral defence (Vance and Vaucheret, 2001; Voinnet, 2001). As an effective countermeasure, several plant viruses have evolved genes that thwart an RNAi response in infected cells (Anandalakshmi et al., 1998; Brigneti et al., 1998; Reed et al., 2003). Recently, an animal virus, flock house virus, able to infect insect cells, was found to encode an inhibitor of the RNAi pathway, indicating that RNAi may play some role in animal antiviral responses (Li et al., 2002). Whether or not RNAi has a role in the mammalian innate immune system's antiviral repertoire remains to be established.

3.3. RNAi in heterochromatin formation, mitosis and meiosis

Components of the RNAi machinery are conserved in unicellular eukaryotes including *Chlamydomonas reinhardtii* (Wu-Scharf et al., 2000), *Trypanosoma brucei* (LaCount and Donelson, 2001) and the fission yeast *Schizosaccharomyces pombe* (Volpe et al., 2002). Apparently, *Saccharomyces cerevisiae* lacks genes with significant homology to RNAi genes identified in other organisms (Aravind et al., 2000). *S. pombe* has one member of the Argonaute family, a Dicer homologue and a gene with potential RDRP activity. Disruption of any of these genes resulted in a loss of heterochromatin formation and loss of centromere function (Volpe et al., 2003), which is essential for faithful chromosome segregation during mitosis (Bernard et al., 2001; Bernard and Allshire, 2002). Thus, RNAi controls gene expression by regulating heterochromatin formation in fission yeasts, which is regulated by histone H3 methylation.

In fission yeast, RNA-directed heterochromatin formation is also involved in the regulation of some meiotic genes. Interestingly, these genes are controlled by the presence of near-by retrotransposon long terminal repeats (LTR), whose transcription induces silencing of the locus. Loss of RNAi-genes or the respective LTRs deregulate the expression of the meiotic genes during vegetative growth (Schramke and Allshire, 2003). Whether LTRs, which are abundantly present in the human genome, control gene expression in humans is not known.

Neurospora crassa is another organism with strong gene silencing mechanisms, one of which can be activated by introduced transgenes. The strong silencing effect of this kind has been termed 'quelling' (Romano and Macino, 1992). In a transgene-induced loss-of-pigmentation screen for quelling defective mutants (*qde*), genes belonging to three complementation groups have been identified (Cogoni et al., 1996). QDE-1 has some similarity to RDRPs (Cogoni and Macino, 1999a), QDE-2 is a member of the Argonaute protein family and *QDE-3* encodes a protein with similarities to the ReqQ helicases, including Bloom's and Werner's syndrome helicases (Cogoni and Macino, 1999b). In contrast to plants, QDE-2 co-purifies with siRNAs (Catalanotto et al., 2002) and is thus likely to function like *Drosophila* AGO2 in the effector step of RNAi (Hammond et al., 2001a).

Unpaired regions of chromosomes during meiosis are another potent signal for gene silencing in *Neurospora* (Hynes and Todd, 2003),which depends on the function of the putative RDRP, SAD-1 (Shiu and Metzenberg, 2002). Interestingly, the SAD-1 related *C. elegans* mutant *ego-1* also displays germline defects and unpaired chromosomes, suggesting that RNAi may be required for meiosis in some species (Smardon et al., 2000).

3.4. RNAi in development

The *Arabidopsis* gene *AGO1*, was initially identified in a genetic screen for developmental mutants, but was later found to have an important role in RNA-induced gene silencing, suggesting that RNAi has an important role in development (Fagard et al., 2000). AGO1 is the founding member of the Argonaute protein family and is homologous to *C. elegans* RDE-1, *Neurospora crassa* QDE-2 and human eIF2C proteins, all of which are required for RNAi.

Studies on worms lacking *DCR-1*, the nematode's orthologue of the *Drosophila* gene *Dicer*, have led to the discovery of yet an additional function of RNAi. These worms lacked RNAi but in addition had a developmental phenotype mimicking the effects of mutations in two small non-coding RNA genes, *lin*-4 and *let*-7 (Grishok et al., 2001). These two heterochronic genes, which are involved in the relative timing of developmental events, are expressed as short temporary RNAs (stRNAs), which are processed into smaller 21-nt-sized RNAs in a Dicer-dependent manner. These stRNAs are similar to miRNAs as they regulate cell fate decisions by controlling the translation of genes.

Since *lin-4* was identified as the first non-coding RNA to be involved in the regulation of development, several other small RNAs have been identified, which influence many

cellular functions (Ambros, 2003). In addition to the conserved genes *lin*-4 and *let*-7 (Pasquinelli et al., 2000), several hundreds of additional miRNAs have recently been cloned in *C. elegans* (Lau et al., 2001), *Drosophila* (Aravin et al., 2003; Lagos-Quintana et al., 2001), mice (Lagos-Quintana et al., 2002) and humans (Lim et al., 2003; Mourelatos et al., 2002), whose functions are largely unknown.

The small non-coding RNA *bantam* has recently been identified in the control of organ size in the fruit fly (Brennecke et al., 2003). Overexpression of *bantam* resulted in excessive tissue growth, while heterozygous mutants were significantly smaller than wild-type animals due to a decrease in cell numbers. Cloning of the *bantam* locus revealed that the gene encodes a novel miRNA. To reveal its function, Cohen and co-workers have developed a computer algorithm to identify putative miRNA targets and identified the pro-apoptotic gene *HID*. *Bantam* regulates the translation of *HID* by binding to complementary sequences in the 3′ UTR of *HID*. These data demonstrate that some miRNAs are important developmental genes by controlling proliferation and apoptosis.

Dicer is an essential gene in *C. elegans* (Ketting et al., 2001; Knight and Bass, 2001) and *Arabidopsis* (Finnegan et al., 2003; Golden et al., 2002) and is also essential in vertebrates. Mice deficient for Dicer die very early in development and appear to lack stem cells as no expression of Oct4, a marker for embryonal stem cells, could be detected (Bernstein et al., 2003). Whether Dicer function is required for a siRNA- or a miRNA-guided effector mechanism is unclear. As embryonic stem cells express many unique miRNAs (Houbaviy et al., 2003), Dicer might be involved in maintaining stem cells. In addition to the proposed function of Dicer, the RNAi pathway has previously been implicated in stem cell maintenance as members of the Argonaute protein family were found to control stem cell fate in various other organisms (Carmell et al., 2002).

Zebrafish deficient in Dicer do not develop normally either, but compared to mouse embryos reach a later stage in embryogenesis because of a maternal contribution of Dicer mRNA and possibly protein. As a consequence of the lack of Dicer, pre-miRNAs accumulate in mutant fish (Wienholds et al., 2003), suggesting that miRNAs, similar to the function of *let-7* and *lin-4* in *C. elegans*, may be essential for vertebrate development.

3.5. RNAi and human disease

Given the multiple roles miRNAs and RNAi play during development, it is likely that mutations in the RNAi pathway may contribute to human disease, although no such association has been proven so far. Of the several Argonaute proteins, which may be involved in the decision of whether dsRNA will be processed into a siRNA or a miRNA, the human *AGO-1* (*eIF2C1*) gene has been linked to Wilm's tumour (Koesters et al., 1999). In addition, the human homolog of *Drosophila* Piwi, *HIWI*, has been associated with the development of seminomas (Qiao et al., 2002). As the RNAi pathway is involved in controlling stem cell fate, high expression of Argonaute proteins might contribute to the development of tumours maintaining stem cell characteristics. A possible involvement of miRNAs has also been postulated by Calin et al. (Calin et al., 2002), who have identified two miRNAs frequently deleted in patients suffering from chronic myeloid leukaemia.

Another example of a possible association of RNAi genes with human disease is fragile X disease, as the FMRP, is a component of the RISC (Caudy et al., 2002). FMRP is found associated with translating polyribosomes in neurons, and is supposed to control the translation of proteins involved in neuronal plasticity and axon guidance (Jin and Warren, 2003). This function is consistent with the role miRNAs play in the control of mRNA translation in other species.

These examples indicate that RNAi is an important pathway that is used for several different purposes from cleaning up aberrant RNAs, removal of viral RNA, control of gene expression and heterochromatin formation. Future studies will shed light on the impact of alterations in the RNAi pathway in causing human disease.

4. RNAi as an experimental tool to study gene function in mammalian cells

As outlined above, cells respond to a dsRNA molecule by shutting down the expression of any gene with high enough homology to the input dsRNA (Hutvagner and Zamore, 2002). Mammalian somatic cells, however, also mount a strong interferon response and activate RNA-dependent protein kinase PKR and RNaseL in response to long dsRNA, which inhibits all protein synthesis and eventually causes apoptosis (Samuel, 2001). This response, however, is not found in embryonic stem cells or teratocarcinoma cell lines and in those cells, long dsRNA can be used to initiate gene specific RNAi (Billy et al., 2001; Wianny and Zernicka-Goetz, 2000; Yang et al., 2001).

Dissection of the RNAi mechanism in *Drosophila* embryo extracts, and demonstration that siRNAs are the effector molecules in RNAi, led Tuschl and coworkers to examine whether siRNAs, which are short enough to bypass an interferon response, are sufficient to trigger RNAi in human cells (Elbashir et al., 2001a). They used in vitro synthesized 21 nt RNA duplexes with 2 nt 3′-overhangs, and transfected them into human tissue culture cells to observe a dramatic and highly specific effect on gene expression (Harborth et al., 2001).

4.1. siRNAs

Since the first description of this technique in 2001, siRNA-mediated RNAi has advanced to one of the most

important and widely used techniques. Synthetic siRNAs are designed by choosing $AAN_{19}TT$ sequences, with a GC content of 50%, located 100–200 nt downstream of the translation initiation codon in the target mRNA. Any candidate sequence has to be analysed by searching for potential homologues among known genes to minimize the chance of unwanted suppression of related, or even unrelated, genes. If the above siRNA design criteria can not be met, it is important to mention that neither the AA, nor the TT flanks are essential nor is the localisation of the siRNA within the coding sequence as functional siRNAs have be derived from 5′UTR, the entire coding sequence, or the 3′ UTR (Harborth et al., 2003).

Following these simple rules, many but not all, of the chosen sequences will efficiently silence their target gene. In an attempt to improve siRNA design rules, Khvorova et al. (2003) have compared efficient with less efficient siRNA sequences used in mammals, worms and flies and discovered that the 5′ end of the antisense, or guide, strand has a lower stability than its 3′ end. This unique feature was also discovered by Schwarz et al. (2003), who have found that destabilising the 5′ end of the antisense strand converts a poor siRNA oligo into a potent one. These studies suggest that an asymmetric design of the siRNA oligo with a high stability 3′ and low stability 5′ end of the antisense strand will enhance silencing efficacy. In accordance with these papers, Aza-Blanc et al. (2003) have described a high frequency of AT rich 5′ antisense strand sequences in efficient siRNA duplexes.

Depending on the experimental needs, synthetic siRNAs can be modified in several ways without losing their efficacy. As a rule, the sense strand of the siRNA molecule can be modified readily, while modification of the antisense strand can render the siRNA inactive. For example, labelling of either the 5′ or 3′ end of the sense strand with fluorescent fluorochromes does not affect the efficacy of a siRNA, while only the 5′ end of the antisense strand can be labelled. It is also possible to modify the backbone structures of the nucleotides, e.g. by using phosphothioate linkages to enhance the stability of the dsRNA, without significantly impairing silencing activity (Harborth et al., 2003).

In addition to chemical synthesis (Pitsch et al., 2001; Scaringe, 2001), siRNAs can be obtained by in vitro transcription using bacteriophage RNA polymerases and short DNA oligonucleotides containing phage promoters as templates (Donze and Picard, 2002). Due to the sequence constraints required for efficient transcription in vitro, the first nucleotides of the short RNA cannot be chosen freely, and, for example, will have to be a G in the context of a T7 promoter. Several methods, including the use of RNases or ribozymes (Sohail et al., 2003) have been devised to overcome these limitations to allow more flexibility in choosing a target sequence. In vitro transcription of siRNAs is an effective, cheap and fast alternative to commercial synthetic siRNAs. This method is also well suited to rapidly determine effective siRNA sequences.

In addition, siRNAs can be generated by digestion of long dsRNA using either recombinant bacterial RNase III (Yang et al., 2002) or human Dicer (Myers et al., 2003; Zhang et al., 2002). Long dsRNA can effectively be obtained after PCR-amplification of a ~500 bp cDNA fragment using primers containing phage promoter sequences as the template for an in vitro transcription reaction. Because many different siRNAs are generated and can be tested in a single experiment, this approach is very useful for setting up a gene-silencing project. It is recommended, however, to confirm data using sequence-defined siRNAs to rule out any unwanted, non-specific effect on other genes.

The success of using siRNAs for gene-silencing experiments depends on the transfection efficiency that can be obtained in a certain cell type. In addition, cell type specific differences in silencing efficiency have been reported (Harborth et al., 2003). Transient transfection of siRNAs is self-limited, and after the maximum effect of RNAi after 2–3 days, cells tend to recover within a week.

4.2. shRNAs

To overcome the limitations of poor transfection efficiency and transient RNAi, several in vivo siRNA expression systems have been devised. The majority of these systems employ RNA polymerase III-dependent type III promoters (Paule and White, 2000), such as the U6 or H1 RNA gene promoters (Brummelkamp et al., 2002a; Miyagishi and Taira, 2002; Paddison et al., 2002a; Paul et al., 2002). Transcription from these small and compact promoters starts at a defined site and can be precisely terminated by using a stretch of 5 consecutive Ts. In contrast to the U6 promoter, where the first nucleotide has to be a G, the H1 promoter seems to tolerate any nucleotide at the +1 position. The two strands of the siRNAs can be expressed individually (Miyagishi and Taira, 2002) or as a short hairpin RNA (shRNA) (Brummelkamp et al., 2002a; Paddison et al., 2002a; Paul et al., 2002).

shRNA expression plasmids are a powerful means to induce stable and even inducible RNAi in mammalian cells. The hairpin RNAs are usually designed as (19–29 nt) sense-loop-(19–29 nt) antisense molecules followed by 5 Ts. These RNAs fold up as hairpins and are processed by Dicer into mature siRNAs. There is some controversy whether the length of the stem (Paddison et al., 2002a) or the structure of the loop (Brummelkamp et al., 2002a) is more important to obtain effective siRNAs. In a recent paper, Tuschl and coworkers analysed in vitro synthesised hairpins for their silencing efficiency and found that a simple 5 nt loop is sufficient and that 21–23 nt long sense and antisense stem sequences are required for effective silencing (Harborth et al., 2003). As with siRNAs, the efficacy of shRNAs is not predictable and has to be determined experimentally.

Due to their small size, shRNA expression cassettes have been incorporated into many different vector

systems, including retro- (Barton and Medzhitov, 2002; Brummelkamp et al., 2002b; Paddison et al., 2002b), lenti- (Rubinson et al., 2003), and adenoviral constructs (Xia et al., 2002) for efficient gene delivery. Retroviral constructs, as well as plasmid integration have been used to generate stable RNAi. Excitingly, stable RNAi has been used to target a mutant version of the *K-Ras* oncogene in a human pancreatic carcinoma cell line, without affecting the expression of wild-type *K-Ras*. Cell lines with RNAi-induced suppression of activated *K-Ras* lost their ability to form colonies in soft agar and tumorigenicity in mice (Brummelkamp et al., 2002b). These results impressively demonstrate the power of RNAi and its potential use as a therapeutic agent. Another striking example was the use of stable RNAi to suppress p53 function to restimulate growth in senescent mouse fibroblasts (Dirac and Bernards, 2003).

4.3. Inducible and transgenic RNAi

Several inducible RNAi systems have been described to allow the use of stable RNAi technology for essential genes. This has been achieved with varying success by rendering the polymerase III dependent promoters sensitive to inducible DNA binding proteins, such as bacterial tetR or tetR fusion proteins (van de Wetering et al., 2003; Wiznerowicz and Trono, 2003).

Additional shRNA expression systems have been developed, which are based on RNA polymerase II-dependent promoters. Shinagawa and Ishii (Shinagawa and Ishii, 2003), for example, have shown that a CMV-promoter driven shRNA is effective and sufficient to induce a loss-of-function phenotype in transgenic mice. This strategy employs a long hairpin RNA (~500 nt), which becomes de-capped by a cis-acting ribozyme and is not polyadenylated. Termination of transcription is achieved by using a transcriptional pause site derived from the MAZ zinc finger protein gene. As many versions of tight conditional polymerase II dependent promoters have been developed, this approach might be promising to develop effective conditional RNAi systems.

RNAi-induced gene silencing has also been achieved in transgenic mice by using integration of shRNA expression plasmids (Carmell et al., 2003; Kunath et al., 2003), and lentiviral (Rubinson et al., 2003) transduction. Because RNA-induced gene-silencing has a knockdown rather than a knockout effect, it is likely that transgenic RNAi will result in less severe, hypomorphic, phenotypes compared to conventional gene-targeting. Nevertheless, transgenic RNAi in mice, like in *C. elegans*, plants or *Drosophila*, is able to phenocopy a true genetic knockout. In addition, RNAi based technologies are useful tools for in vivo reconstitution experiments, e.g. regeneration of the hematopoietic system using stem cells expressing shRNAs (Hemann et al., 2003).

siRNA- or shRNA-induced RNAi has been shown to be very specific with a single nucleotide mismatch greatly reducing the RNAi effect on selected target genes (Elbashir et al., 2001c; Martinez et al., 2002b). To investigate the specificity of these reagents, several groups have used expression profiling experiments to look for off-target gene effects of siRNAs transfected into human tissue culture cells. While Chi et al. (2003) and Semizarov et al. (2003) find little unspecific effects on overall gene expression patterns, Jackson et al. (2003) draw a more dire conclusion on the specificity of siRNAs. They showed that limited sequence homology of the 3′ end of the antisense strand to other, even unrelated, genes could cause the rapid degradation of their mRNAs in transient transfection experiments. Therefore, when designing gene specific siRNAs, care should be taken to avoid siRNA sequences which display complementarity at their antisense 3′ ends to any other than the target mRNA sequence.

As the specificity of siRNAs can not be predicted by gene homology-based data base searches, vigorous controls should be carried out to prove the target gene specific effect of these reagents. Stringent controls for gene specific effects would include the use of siRNAs with a 2–3 nt central mismatch to the target sequence. In addition, overexpression of RNAi resistant versions of a certain cDNA, i.e. a cDNA harbouring 2–3 silent mutations in the siRNA target region, should be used to prove that the observed phenotype can be ascribed to the targeted gene only.

5. Towards RNAi medicine?

RNAi is an established method to dissect the function of genes in various model organisms, such as *C. elegans* and *Drosophila*, and has been demonstrated to be a highly specific and potent means to interfere with the expression of individual genes in human cells. As already mentioned, allele specific RNAi disrupted the function of an oncogene (Brummelkamp et al., 2002b) and was used to suppress the expression of a dominant allele causing amyotrophic lateral sclerosis (Ding et al., 2003). These results suggest that RNAi-based therapeutics could be used to repress the function of genes, such as activated oncogenes, dominant disease alleles or viral genes.

The established role of RNAi in the control of viral infection in plants (Ratcliff et al., 1999) may pertain to animal cells as well (Li et al., 2002). Several viral genes have been identified, which function to circumvent control by RNAi. To investigate whether human viruses may be targeted by RNAi, several studies have been reported, which efficiently interfered with the replication of viruses in human cells. In addition, as these RNAi-based strategies could be applied in adult mice to prevent viral disease (reviewed in Gitlin and Andino, 2003; McCaffrey et al., 2002), RNAi-therapeutics might be effective to fight viral infections, such as AIDS. It will be important to uncover any contribution of RNAi to the human

innate immune response against viruses, as well as the investigation of any RNAi-inhibitory genes encoded in viral genomes.

Another important issue concerning the specificity of RNAi is to evaluate whether mammalian cells, tissues or organisms are capable of transitive RNAi. Transitive RNAi bears the risk that specificity may be lost if the RNAi response spreads into flanking sequences that are shared among different genes. Before RNAi can enter clinical trial, it has to be ruled out that human cells can initiate transitive RNAi, for which no evidence has been obtained thus far (Chi et al., 2003; Schwarz et al., 2002). Although transfer of RNAi between cells has not been observed in human tissue culture cells, systemic RNAi should be given serious considerations to avoid the unwanted spread of RNAi inducing agents throughout the organism. Induction of an interferon response has also to be ruled out, which can be induced by siRNAs (Sledz et al., 2003) as well as shRNAs (Bridge et al., 2003).

Even if gene specific siRNAs can be generated and transitive as well as systemic RNAi can be ruled out in humans, several important issues remain to be solved. As outlined above, RNAi has several important physiological functions ranging from maintaining chromatin structure in fission yeast (Bailis and Forsburg, 2002; Dernburg and Karpen, 2002), silencing of transposable elements in *C. elegans* and *Drosophila*, antiviral defence (Gitlin and Andino, 2003), to development in invertebrates and vertebrates. It is unclear which of these functions are conserved in humans, but as Dicer is an essential enzyme in vertebrates, and hundreds of miRNAs have been identified, it is likely that the RNAi pathway has important physiological functions.

It is, therefore, critical to know if the RNAi machinery can be saturated by applying siRNAs for a gene silencing effect, because one might expect serious long-term consequences on the cells or the treated organism. It has been shown that siRNAs can compete with each other for silencing effects, indicating that incorporation into RISCs may be rate limiting (Kamath et al., 2000). Long-time effects of siRNA-induced RNAi are still lacking but will have to be investigated to assess whether RNAi will become a useful therapeutic approach to modify gene expression. Finally, the problem of cell targeting will be an issue in RNAi-based therapies, as it is with every attempted gene therapy.

6. Conclusions

RNAi technology is developing rapidly, and although fairly young, has already become an essential experimental tool. Because the degree of RNAi-induced knockdown effects is often sufficient to study the function of a gene, reverse genetics using siRNAs can be used to probe the function of many hundreds to thousands of genes and is used for functional genomics in various species (Fraser et al., 2000; Gonczy et al., 2000; Kamath et al., 2003), including humans (Aza-Blanc et al., 2003; Berns et al., 2004; Brummelkamp et al., 2003). Compared to its widespread and effective use, however, we know relatively little about the physiological functions of the RNAi pathways. Elucidation of miRNA function and the role of enzymes involved in RNAi will shed new light on the function of this pathway in humans and whether it is involved in human disease. A detailed analysis of RNAi in humans is also mandatory should RNAi-based therapeutics ever be applied in humans to influence the expression of certain genes, such as activated oncogenes or viral RNAs.

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Note added in proof

During the preparation of the manuscript another paper has been published using a RNAi library to investigate the function of the human proteasome proving the efficacy of siRNA-based functional genetics in human tissue culture cells (Paddison PJ. Silva JM, Conklin DS, Schlabach M, Li M, Aruleba S, Balija V, O’Shaughnessy A, Gnoj L, Scobie K, Chang K, Westbrook T, Cleary M, Sachidanandam R, McCombie WR, Elledge SJ, Hannon GJ., 2004. A resource for large-scale RNA-interference-based screens in mammals. Nature 428: 427–431.

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