RNA Interference: Past, Present and Future

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Abstract
RNA interference (RNAi) is the sequence-specific gene silencing induced by double-stranded RNA. RNAi is mediated by 21-23 nucleotide small interfering RNAs (siRNAs) which are produced from long double-stranded RNAs by RNAse II-like enzyme Dicer. The resulting siRNAs are incorporated into a RNA-induced silencing complex (RISC) that targets and cleaves mRNA complementary to the siRNAs. Since its inception in 1998, RNAi has been demonstrated in organisms ranging from trypanosomes to nematodes to vertebrates. Potential uses already in progress include the examination of specific gene function in living systems, the development of anti-viral and anti-cancer therapies, and genome-wide screens. In this review, we discuss the landmark discoveries that established the contextual framework leading up to our current understanding of RNAi. We also provide an overview of current developments and future applications.

Introduction
RNA interference (RNAi), initially coined by Fire *et al.*, (1998), is the sequence-specific gene silencing induced by double-stranded RNA (dsRNA; Figure 1; Wall and Shi, 2003). This phenomenon has generated an explosion of interest and enthusiasm by scientists, similar to that created by the entrance of the green fluorescent protein as a species-independent molecular reporter in the mid-1990s. Since its inception, RNAi has been induced in organisms ranging from trypanosomes (Ngo *et al.*, 1998) to nematodes (Fire *et al.*, 1998) to flies (Kennerdell and Carthew, 1998) to vertebrates (Wianny and Zericka-Goetz, 2000). Because of its specificity, efficiency, and cost-effectiveness, RNAi has drawn the bulk of attention away from previous antisense methods such as ribozymes and oligodeoxynucleotides (ODNs). Furthermore, technical expertise accumulated from previous approaches is now being applied to RNAi, thus rapidly advancing its application to the medicinal domain (Kim, 2003). Currently, three areas of human disease research have already strongly embraced the RNAi phenomenon: infectious disease, cancer, and dominantly inherited disorders (Kim, 2003; Wall and Shi, 2003). RNAi has also shown exceptional promise as a genome-wide screening tool (Kamath *et al.*, 2003; Kiger *et al.*, 2003; Berns *et al.*, 2004; Paddison *et al.*, 2004).

In this review, we discuss the landmark discoveries that established the contextual framework leading up to our current understanding of RNAi. We also provide an overview of current developments and future applications.

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RNAi: Past
Prior to the discovery of RNAi, several other previously characterized, homology-dependent gene silencing mechanisms had been described. In 1990, the Jorgensen laboratory introduced exogenous transgenes into petunias in an attempt to upregulate the activity of a gene for chalcone synthase, an enzyme involved in the production of specific pigments (Napoli *et al.*, 1990; Agrawal *et al.*, 2003). Unexpectedly, flower pigmentation did not deepen, but rather showed variegation with complete loss of color in some cases. This indicated that not only were the introduced trangenes themselves inactive, but that the added DNA sequences also affected expression of the endogenous loci (Hannon, 2002). This phenomenon was referred to as "cosuppression" (Napoli *et al.*, 1990). Reports from other laboratories noted similar effects (Vander Krol *et al.*, 1990; Ingelbrecht *et al.*, 1994). Subsequently, cosuppression was renamed posttranscriptional gene silencing (PTGS) since all cases of cosuppression resulted in the degradation of endogene and transgene RNAs post-nuclear transcription and this posttranscriptional RNA degradation was observed in a wide range of transgenes expressing the plant, bacterial, or viral sequences (Kooter *et al.*, 1999; Agrawal *et al.*, 2003).

As more researchers corroborated PTGS results in plants, a similar phenomenon labelled "quelling" was reported in fungi. Two different laboratories demonstrated transgene-induced silencing of both transgenes and

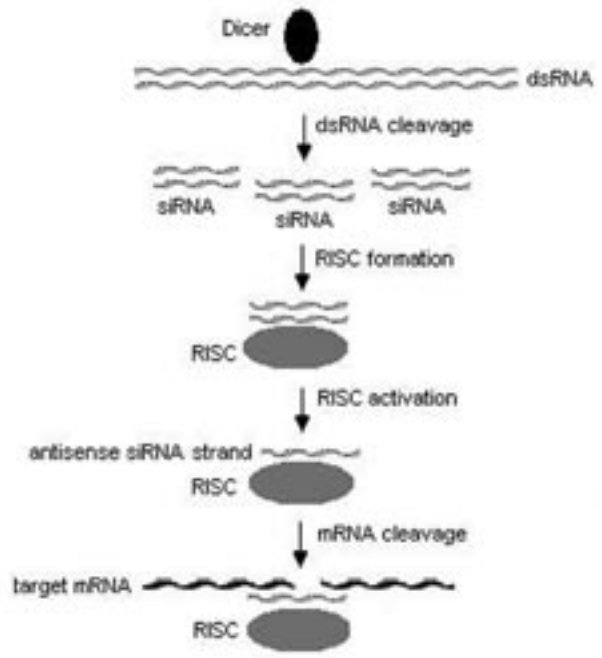

Figure 1. Proposed mechanism of RNA interference. Abbreviations include: dsRNA (double-stranded RNA), siRNA (small interfering RNA), RISC (RNA-induced silencing complex), and mRNA (messenger RNA).

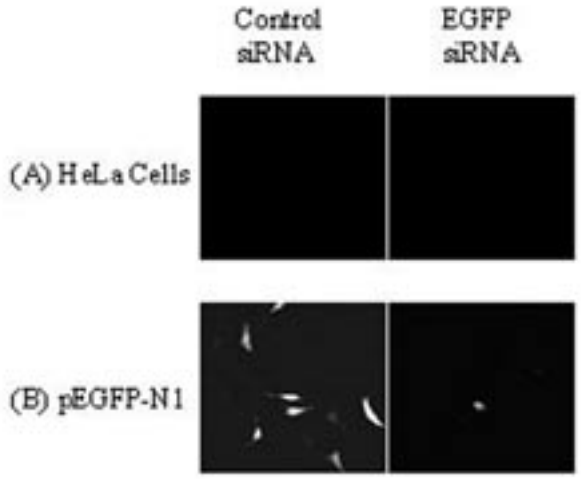

Figure 2. Epifluorescence microscope at 20X magnification in HeLa cells 72 hours following co-transfection with small interfering RNAs (siRNAs). (Campbell and Choy, 2003)

endogenous genes in *Neurospora crassa* (Pandit and Russo, 1992; Romano and Macino, 1992). In 1996, a *N. crassa* strain was transformed with a plasmid containing a segment of the *al1* gene to increase production of an orange pigment. The opposite, however, was observed, with a few stably quelled transformants showing albino phenotypes (Cogoni *et al.*, 1996). These results substantiated earlier observations of transgene-induced gene silencing in *N. crassa*, but also demonstrated that DNA methylation was not obligatory for this process (Cogoni *et al.*, 1996; Sweykowsa-Kulinska *et al.*, 2003).

Around the same time, Guo and Kemphues (1995) demonstrated that sense RNA was as effective as antisense RNA in suppressing gene expression in C*aenorhabditis elegans*. Fire *et al.*, (1998) built upon these results, leading to the discovery of the RNAi phenomenon. The researchers targeted the *unc22* gene, which encodes a nonessential myofilament protein. A decrease in *unc22* activity produces a twitching phenotype. Results indicated that introduction of a dsRNA mixture directed at the *unc22* gene was at least tenfold more effective than were sense or antisense RNA alone. Similar results were noted when other genes were targeted. This experiment paved the way for specific silencing of a functional gene by exogenous application of dsRNA in organisms ranging from worms (Fire *et al.*, 1998) to trypanosomes (Ngo *et al.*, 1998) to flies (Kennerdell and Curthew, 1998). Initial experiments in mammals proved disappointing, since introduction of dsRNA >30 base pairs triggered the interferon response, resulting in global shutdown of protein translation and, ultimately, dramatic alteration in cellular metabolism (Gil and Esteban, 2000). To avoid this general shutdown, Elbashir *et al.*, (2001) chemically synthesized small RNAs which mimicked Dicer products, resulting in the desired gene-specific silencing in mammalian systems. These findings highlighted the possibility of RNAi-induced gene silencing as a therapeutic alternative. Within the same year, Caplen *et al.*, (2001) demonstrated knockdown of enhanced green fluorescent protein (EGFP), a modified version of the *Aequorea victoria* wildtype GFP (Campbell and Choy, 2003; 2004; Figure 2). From this point on, EGFP has been employed as a popular positive control to ascertain successful siRNA delivery and to test system integrity.

RNAi: Present

RNAi Mechanism

Biochemical and genetic studies have confirmed that RNAi, PTGS and quelling share mechanistic similarities and that the biological pathways underlying dsRNA-induced gene silencing exist in many eukaryotic organisms (Hannon, 2002). Due to the explosion of interest in RNAi and its application to many diverse systems, much attention has been focused on elucidating its mechanism of action. Several landmark events, including evidence that siRNAs are the key mediators of RNAi (Hamilton and Baulcombe, 1999) and the cloning of the RNAse III-like enzyme Dicer (Bernstein *et al.*, 2001), have provided insight into the RNAi pathway. Currently, a generalized mechanism has been proposed (Figure 1). First, long dsRNA is processed by Dicer into 21-23 nucleotide siRNAs. These siRNAs are incorporated into a nuclease complex labelled the RNA-induced silencing complex (RISC). The incorporated siRNAs are then unwound, leaving the antisense strand remaining in RISC, resulting in complex activation. The activated RISC now targets and cleaves mRNA that is complementary to the siRNA (Zamore *et al.*, 2000; Kim, 2003; Wall and Shi, 2003). Though this general mechanism has been supported by many laboratories, much research is being performed to gain a better understanding of the fundamental biochemistry and kinetics of the RNAi pathway.

siRNA Design

While there are currently no reliable methods to identify the ideal sequence for a siRNA, a number of parameters have been suggested. These include: selection of a target cDNA region 50-100 nucleotides downstream of the start codon, selection of a 5'-AA(N19)UU target mRNA sequence where N is any nucleotide, 50% G/C content in the target sequence, avoidance of 5' or 3' untranslated regions and high G-rich areas, and confirmation of exclusive target-specific sequences (Elbashir *et al.*, 2002). Presently, custom siRNA synthesis service is available through a number of companies, including Dharmacon, QIAGEN, and Ambion.

The search for more accurate, efficient siRNA selection has resulted in a number of new developments. In order to determine the accessibility of target mRNA sites to better predict optimal siRNA sequences, several groups have developed a synthetic oligodeoxyribonucleotide/RNase H method (Yang *et al.*, 2002; Vickers *et al.*, 2003). These groups discovered a significant correlation between RNase H-sensitive sites and sites that promote efficient siRNA-directed mRNA degradation. In a different approach, a number of groups have employed either *Escherichia coli* RNase III (Calegari *et al.*, 2002; Kawasaki *et al.*, 2003) or recombinant human Dicer (Lee *et al.*, 2002; Myers *et al.*, 2003) to cleave long dsRNA into siRNAs for transfection into mammalian cells, allowing for the introduction of siRNAs with multiple specificities to the target.

siRNA Delivery

A number of different approaches have been used to introduce siRNAs into both cells and whole organisms. Successful approaches include: electroporation (McRobert and McConkey, 2002), soaking in siRNAs (Malhotra *et al.*, 2002), feeding bacteria carrying dsRNA (Caplen *et al.*, 2001), transfection with commercial reagents (Elbashir *et al.*, 2001), and vector-based strategies (Lois *et al.*, 2002; Paddison *et al.*, 2002). Though transfection with commercial reagents is the most widely used RNAi technique at present (Kim, 2003), much focus has been placed on vector-based strategies due to their therapeutic potential. These vector-based strategies involve either DNA or viral vector-mediated RNAi. In the first approach, RNA Polymerase II or III promoters have been incorporated into DNA vectors along with siRNA expression cassettes (Brummelkamp *et al.*, 2002a; Paddison *et al.*, 2002; Sui *et al.*, 2002; Xia *et al.*, 2002). These cassettes have included either sense and antisense siRNA strands expressed from tandem promoters or a short hairpin (shRNA) cassette whereby the two siRNA strands are separated by a short spacer (Dykxhoorn *et al.*, 2003). Use of RNA Polymerase II promoters to generate shRNAs has the advantage that they permit easier adaptation of inducible/repressible tissue- or cell-specific siRNA expression (Wall and Shi, 2003).

Since plasmid-based siRNA expression has limitations in cases where transfection efficiency is low, viral vectors have also been developed. Adenoviral vectors and various retroviral vectors (e.g. lentivirus-based) have proven effective delivery systems in numerous cell types including non-cycling cells, stem cells, and zygotes (Abbas-Terki *et al.*, 2002; Lois *et al.*, 2002; Rubinson *et al.*, 2003; Shen *et al.*, 2003).

RNAi Medical Applications

Soon after the discovery of RNAi, its potential application to therapeutics received much attention. Three main areas of human disease have especially embraced the idea of RNAi therapy: infectious disease, cancer, and dominantly inherited genetic disorders (Kim, 2003). Regarding viral infection, human immunodeficiency virus (HIV), hepatitis, and influenza have all been successfully targeted by RNAi techniques. HIV *tat, rev, nef* and *gag* genes have been silenced, resulting in inhibition of viral replication in cultured cells (Lee *et al.*, 2002; Park *et al.*, 2002; Boden *et al.*, 2004; Das *et al.*, 2004). In mice, reduction in hepatitis B virus RNA and replicative intermediates has been demonstrated upon introduction of siRNAs or shRNA vectors (Giladi *et al.*, 2003; McCaffrey *et al.*, 2003). Likewise, the accumulation of influenza viral mRNAs was arrested following addition of siRNAs specific for nucleocapsid or a component of the RNA transcriptase (Ge *et al.*, 2003).

In cancer research, RNAi has been used to target dominant mutant oncogenes, amplified oncogenes, translocation products, signalling molecules, and viral oncogenes (Cheng *et al.*, 2003). For example, through the use of an RNAi retroviral system targeting K-RAS in human pancreatic carcinoma cells, Brummelkamp *et al.*, (2002b) were able to induce loss of anchorage-independent growth and tumorigenicity. In another example, RNAi was used to knock down the MDR1 gene product P-glycoprotein, thus reducing cancer cell multidrug resistance (Wu *et al.*, 2003).

Researchers in the area of dominantly inherited disorders have also successfully employed RNAi techniques (Caplen *et al.*, 2002; Xia *et al.*, 2002; Abdelgany *et al.*, 2003; Miller *et al.*, 2003). Such disorders are particularly amenable to RNAi since targeted silencing of the mutated allele would leave the wildtype allele to restore the normal cellular function. Miller *et al.*, (2003) took advantage of a single-nucleotide polymorphism to generate a siRNA that selectively silenced the mutant Machado-Joseph disease/spinocerebellar ataxia type 3 allele without suppressing the wildtype. Allele-specific silencing has been also been demonstrated in mammalian cells expressing a pathogenic mutant acetylcholine receptor (AChR) subunit characteristic of slow channel congenital myasthenic syndrome (Abdelgany *et al.*, 2003). Transfection of either siRNAs or shRNAs resulted in efficient discrimination between the knockdown of the AChR mutant and the wildtype.

RNAi Genomic Applications

Genome-wide RNAi surveys of gene function were initially reported in *C. elegans* (Kamath *et al.*, 2003). Since then, the RNAi library created from this early model has been replicated and reused to screen for genes involved in nematode body fat regulation (Ashrafi *et al.*, 2003), longevity (Lee *et al.*, 2003), and genome stability (Pothof *et al.*, 2003). Not surprisingly, RNAi has also been adapted as a large-scale screening tool in another popular model system, *Drosophila* (Kiger *et al.*, 2003). Recently, Paddison *et al.*, (2004) and Berns *et al.*, (2004) reported the generation of tools to permit RNAi mass-screening of mammalian genes. Both groups generated a retrovirus-based library with multiple cloned shRNAs for most of the targeted genes. Paddison *et al.*, (2004) demonstrated the effectiveness of their library with a screen for human proteosome defects, while Berns *et al.*, (2004) used their library to identify one known and five novel components of the p53 signalling pathway.

RNAi: Future

Since its initial discovery in 1998 by Fire *et al.*, RNAi has taken the scientific community by storm. Despite many rapid advances, however, RNAi is still in its infancy (Table 1). Though Song *et al.*, (2003) successfully demonstrated the therapeutic use of siRNAs to treat fulminant hepatitis in mice, other researchers have shown that certain siRNAs may still trigger the interferon response (Bridge *et al.*, 2003; Sledz *et al.*, 2003). Several factors could account for this response, including siRNA sequence/quality, use of invasive transfection reagents, or differences in cell lines and culture conditions (Frantz, 2003). Nevertheless, caution should be taken when evaluating siRNA effectiveness as a therapeutic agent.

A better understanding of the components and mechanism of the RNAi pathway is a future goal shared by many laboratories. Knowledge gained from the RNAi-dependent chromatin-based silencing pathway reported in the fission yeast *Schizosaccharomyces pombe* may provide insight into similar pathways in plants and animals (Schramke and Allshire, 2003; Matzke and Matzke, 2003). This may also lead to improved methods and guidelines for siRNA sequence selection, which are necessary to

Table 1. Landmark events in the discovery and development of RNA interference (RNAi).		
Year	Event	Reference
1998	First description of RNAi; Silencing heritable in *C. elegans*	Fire *et al*.
1999	First evidence of siRNAs as the key mediators of RNAi	Hamilton and Baulcombe
2000	Mechanism of RNAi beginning to be defined	Zamor *et al*.
2000	Genes involved in RNAi and RNAi inheritance beginning to be assembled	Grishok *et al*.
2001	Cloning of Dicer	Bernstein *et al*.
2001	First evidence that siRNAs can mediate sequence-specific gene silencing in mammals	Elbashir *et al*. (a)
2001	First EGFP siRNA designed (used as a standard control thereafter)	Caplen *et al*.
2001	siRNA design rules suggested	Elbashir *et al*. (b)
2002	siRNA plasmid and viral vectors created for mammalian siRNA expression	Brummelkamp *et al*., Lois *et al*, Paddison *et al*., Sui *et al*.
2003	Therapeutic siRNAs used successfully in whole animals	Song *et al*.
2003	RNAi used in functional genomic approach	Kamath *et al*.
2003	RNAi shown to influence heterochromatin formation	Schramke and Allshire
2003	Certain siRNAs shown to trigger interferon response	Sledz *et al*., Bridge *et al*.
2004	Large-scale mammalian RNAi screens	Paddison *et al*., Berns *et al*.

avoid current trial-and-error tribulations. Furthermore, 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. Finally, the generation of additional RNAi protocols for genome-wide screens will assist with the rapid identification of genes involved in specific biological processes. Thus, though the promise of RNAi is yet to be fulfilled, its potential is beginning to be realized.

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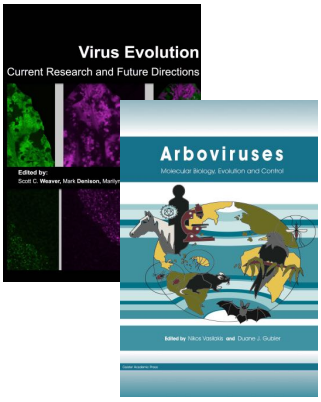

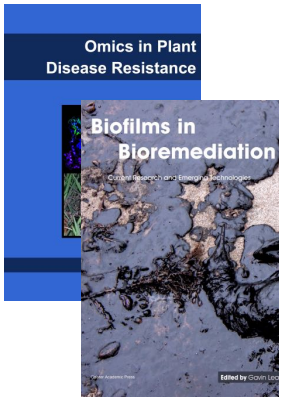

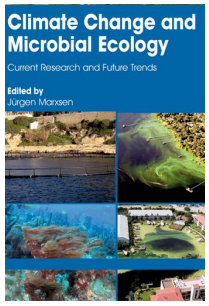

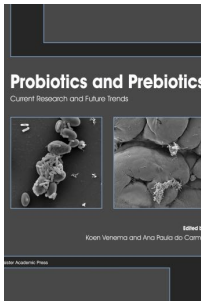

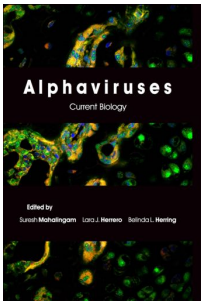

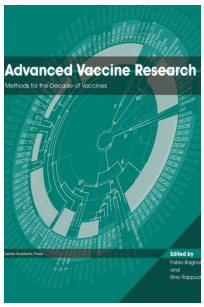

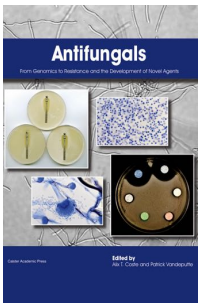

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