CANCER GENOMICS & PROTEOMICS *3*: 127-136 (2006)

Review

RNA Interference with siRNA

HELENA JOYCE*, ISABELLA BRAY* and MARTIN CLYNES

National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin, Ireland

Abstract. *Over the last decade, RNA interference has emerged as an effective mechanism for silencing gene expression. This ancient cellular antiviral response can be used to allow specific inhibition of the function of any chosen target gene, including those involved in diseases such as cancer, AIDS and hepatitis. It has become an invaluable research tool to aid in the identification of novel genes involved in disease processes. It has advanced from the use of synthetic RNA for the endogenous production of small hairpin RNA by plasmid and viral vectors, and from transient inhibition in vitro to longer-lasting effects in vivo. However, as with antisense and ribozymes, the efficient delivery of siRNA into cells is currently the limiting factor to successful gene expression inhibition in vivo. This review gives an overview of the mechanism of action of siRNA and its use in cancer research. It also discusses the successes and shortcomings of this new gene knockdown tool.*

siRNA – A Brief History

RNA silencing was discovered in plants more than 15 years ago during the course of transgenic experiments that eventually led to silencing of the introduced transgene and, in some cases, of homologous endogenous genes or resident transgenes (1-5). Between 1998 and 2001, RNAi was observed in yeast (6), *Drosophila* (7), insects (8), planarians (9) and trypanosomes (10). RNAi was also compared to post-transcriptional or homology-dependent gene silencing (PTGS) in plants (11-12), raising the possibility that RNAi was a mechanism present in all eukaryotes. The year 2001 saw the first successful siRNA experiments in mammalian cells using RNA interference mediated by duplexes of 21-nt RNAs (13). Twenty-one different gene products with different functions and subcellular localizations were studied. Knockdown experiments, monitored by immunofluorescence and immunoblotting, showed that even major cellular proteins such as actin and vimentin could be silenced efficiently. Previous work had discovered that these 22-nucleotide sequences served as guide sequences that instruct a multicomponent nuclease, RNA-induced silencing complex (RISC), to destroy specific messenger RNAs (7). It was then found that, in response to dsRNA, cells trigger a two-step reaction. In the first step, long dsRNA is processed by a ribonuclease (RNase) III enzyme, called Dicer, into small interfering RNAs (siRNAs); these subsequently serve as the sequence determinants of the RNAi pathway by directing cleavage of homologous mRNA *via* a RISC. Dicer is a member of the RNase III family of nucleases that specifically cleave double-stranded RNAs, and is evolutionarily conserved in worms, flies, plants, fungi and mammals (14). The most recent development in this area is the use of RNAi-based therapies in phase I clinical trials, which have been carried out over the past 12 months.

In the region of 15 RNAi papers were published in 1998, whereas over 1,000 were published in 2005. Many groups have changed their focus from antisense and ribozyme technologies to concentrate on post-transcriptional gene silencing using RNA. (15).

Mechanism of Action – An Overview

Long double-stranded RNAs (typically >200 nt) can be used to silence the expression of target genes in a variety of organisms, as already mentioned. Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. During the initiation stage, long dsRNA is cleaved into siRNA and miRNAs (16), mediated by type III RNase Dicer. RNase III family members are among the few nucleases that show specificity for dsRNAs (17) and are evolutionarily conserved in worms, flies, fungi, plants and mammals (18). Complete digestion by the RNase III enzyme results in dsRNA fragments of 12 bp to 15 bp, half the size of siRNAs (19),

*Both authors contributed equally to this work.

Correspondence to: Helena Joyce, National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland. Tel: 00 353 1 7005700, Fax: 00 353 1 7005484, e-mail: helena.joyce@dcu.ie

Key Words: RNA interference, siRNA, microRNA, cancer, review.

however examination of the crystal structure of the RNase III catalytic domain explains the generation of 23- to 28-mer diced siRNA products (20). In this model, Dicer folds on the dsRNA substrate to produce two active catalytic sites having homology with the consensus RNase III catalytic sequence, and two inactive internal sites. The diced products are the limit digests and are double the size of the normal fragments.

During the effector stage, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process (7). Dicers are part of the RISC complex, which includes several different proteins such as the Argonaute gene family members and an ATP-dependent RNA helicase activity that unwinds the two strands of RNA. Argonaute proteins contain two RNA-binding domains, one that binds the small RNA guide at its 5'-end, and one that binds the single-stranded 3'-end of the small RNA. The siRNA must be 5'-phosphorylated to enter the RISC complex. The antisense strand is exposed by the helicase and only one strand of the siRNA guides the RISC to the homologous strand of the target mRNA. Functional RISCs contain only single-stranded siRNA or miRNA (21). The siRNA strands subsequently guide the RISC to complementary RNA molecules, where Watson and Crick base-pairing takes place between the antisense strand of the siRNA and the sense strand of the target mRNA. This leads to endonuclease cleavage of the target RNA at the phosphodiester bond, 10 to 11 nucleotides along from the 5'-end of the siRNA (22). Gene silencing by RISC is accomplished *via* homology-dependent mRNA degradation (23, 24), translational repression (25) or transcriptional gene silencing (26).

Endonucleolytic cleavage is generally favoured by perfect base-pairing between the miRNA /siRNA and the mRNA, although some mismatches can be tolerated and still allow cleavage to occur (27, 28). Translation repression is seen mostly in miRNA, though evidence of siRNA acting as miRNA does exist (29). A short RNA with mismatches to a target sequence present in multiple copies in the 3'-untranslated region (UTR) of an exogenously expressed gene can silence it by translational repression. A single base mismatch with the target is believed to protect the mRNA from degradation, making this type of interference highly specific to the targeted gene (30). Transcriptional gene silencing causes gene expression to be reduced by a blockade at the transcriptional level. Transcriptional silencing by siRNAs probably reflects genome defence mechanisms that target chromatin modifications to endogenous silent loci such as transposons and repeated sequences (29, 31).

siRNA/miRNA - what's the difference?. RNAi regulation of endogenous genes in mammalian cells occurs *via* production of short double-stranded RNA molecules termed microRNA or miRNA. miRNAs are a class of non-coding RNAs that function as endogenous triggers of the RNAi interference pathway (32). Mature miRNAs are between 21 to 23 nucleotides in length and are formed from larger transcripts, 60-80 nt. These long precursors are produced by RNA polymerase II, spliced, polyadenylated and resemble mRNAs (though they may or may not have an open reading frame (ORF)). First, the larger transcripts fold to produce hairpin structures that are substrates for the RNase III enzymes, Drosha, located in the nucleus. This functional stem-loop structure can be located in an intron or an exon. Following this initial processing, pre-miRNAs are escorted through the nuclear pore by exportin-5, a transport receptor (33). As with siRNA, the pre-miRNA is now processed by Dicer to form mature miRNA duplexes (34).

siRNA refers to the synthetic generation of RNA interference. siRNA are 21 to 22 nucleotides in a staggered duplex, with two unpaired nucleotides at either end and are perfectly complementary to their target sequence, causing silencing at the mRNA level. miRNA, on the other hand, possess a strand which is highly, but not perfectly, complementary to one or more target mRNAs. This causes the assembly of an mRNA-protein complex on the target mRNA, preventing translation (35).

miRNA in cancer. Recent studies of miRNA expression implicate miRNAs in brain development (36), chronic lymphocytic leukemia (37), colonic adenocarcinoma (38), Burkitt's lymphoma (39) and viral infection (40), suggesting possible links between miRNAs and viral disease, neurodevelopment and cancer. miRNA has been shown to act as both tumour suppressors and oncogenes. More than 50% of miRNA genes have been found localized in cancer-associated genomic regions or in fragile sites (37). Expression profiling methods were developed to analyse 217 mammalian miRNAs from a panel of 200 human cancers. The results showed an overall reduction in expression of miRNAs in cancer compared to normal samples. This suggests that miRNAs may act predominately as tumour suppressors (41, 42). However, a cluster of miRNAs, miR-17 ~ 92, is overexpressed is some lymphoma and solid tumours. Ectopic expression of these miRNAs in a mouse model of Burkitt's lymphoma led to accelerated and disseminated disease (43).

RNAi in Cancer Research

Experimental considerations in vivo and in vitro. RNAi is a powerful tool for the generation of tissue culture or animal models with reduced expression of specific genes. However, before embarking on *in vivo* studies using RNAi, many important factors need to be taken into consideration. These include site selection, compound design, controls, route of administration and use of a delivery system (44). It

is probable that many adverse effects will be observed *in vivo* using siRNA that may not have occurred in previous experiments using antisense and ribozymes. It is important to be aware of the lifespan of the chosen RNAi in *in vitro* and *in vivo* experiments. Intracellular degradation of siRNA peaks around 36 to 48 h after their introduction and begins to decrease after 96 h. The levels of silencing vary between species, cells and tissues due to differences in the efficiency with which the siRNAs are taken up by the target cells. The duration of gene silencing varies greatly between cells, with slow growing cells still showing the effects of siRNA after several weeks, but more rapidly dividing cells not seeing an effect for longer than 1 week (45). Also, the targeting of proteins with a long half-life may not produce the desired phenotypic effect, because silencing at the level of transcription will not effect pre-existing proteins. Therefore, RNAi has the optimal effect in proteins with a more rapid turnover (46).

shRNA and siRNA libraries. These vectors contain RNA polymerase III promoters that either express sense and antisense strands from separate promoters (tandem type), or express short hairpin RNAs (shRNAs) that are cleaved by Dicer to produce siRNA (shRNA). Stably transfected cell lines can be generated by selecting for a drug resistance marker (expressed either on the vector or on a co-transfected plasmid). Such vector systems have been successfully used to obtain the efficient and stable knockdown of target genes in mammalian cells (47). Research has shown that the shRNA system is more effective than the tandem system (48). In addition, a recent study indicated that shRNAs are more potent inducers of RNAi than is siRNA (49).

As with antisense technology, the delivery method is one of the key challenges for the use of siRNA, especially for delivery to primary cells and *in vivo*. The use of viral delivery vectors has enabled the more efficient delivery of siRNA into a wider spectrum of cells than is possible with transfection technologies. When expressed from viral vectors, the expression of shRNAs has been shown to be long-term (over a month or more). Several groups have built siRNA libraries (viral or non-viral) to elucidate the functions of genes in various biological processes.

The concept of down-regulating a single gene by siRNA can be exploited by high-throughput technologies to facilitate large-scale genomic studies. This can be done in a microtiter-based format or by designing libraries of siRNA or shRNA molecules. Libraries may be engineered based on validated siRNA sequences, or by using randomly produced sequences and a biochemical or cellular assay to identify positive elements. If multiple iterations are used, the siRNA or shRNA may be rescued, re-introduced and identified by sequencing. This strategy may be effectively used to identify drug targets or new components of signalling pathways.

Large collections of synthetic siRNA targets are now available for the screening of targets. Screening is typically carried out in a 96-well format, with each well containing multiple siRNA species. These RNAi screens are now widely used in cancer research to identify both tumour suppressors and oncogenes. 'Positive' screens rely on the ability of an interfering RNA to rescue a cell from some cytotoxic or cytostatic influence, and are ideal for the identification of tumour suppressor genes. In contrast, a 'negative' screen identifies genes by producing a cytotoxic or cytostatic phenotype. One study used such libraries to screen for siRNAs that synergise with other apoptotic stimuli in cell killing. The work produced an extensive list of prospective chemotherapy targets, several of which were individually confirmed (50). Ngo *et al.* (51) described a loss-of function screen for genes required for the proliferation and survival of cancer cells using an RNAi library. In this case, a doxycycline-inducible retroviral vector for the expression of small hairpin RNAs (shRNAs) was used to construct a library targeting 2,500 human genes. This system allowed comparison of cells with and without shRNA expression, while also taking into consideration the delivery of shRNA and the effects of the non-expressing vector. Each shRNA was tagged and the relative abundance in doxycycline-positive and -negative populations monitored using microarray (51). The result was the discovery of CARD11 as a key upstream signalling component responsible for the constitutive Ikβ kinase activity in activated B-cell-like DLBCL (diffuse large B-cell lymphoma). The methods described in this study could be useful in establishing a functional taxonomy of cancer and assist in the discovery of new classes of therapeutic targets distinct from known oncogenes.

The main limitation of siRNA libraries is that, because they are based on the sequence information of previously recognized mRNAs, they are unable to identify previously unknown genes. Expression libraries, on the other hand, use collections of siRNAs of known sequence, but unknown target. The idea is to identify genes with specific biological properties. For example, siRNA, which promotes an oncogenic phenotype, does so by targeting tumour suppressors. One such library was used to identify genes involved in anchorage-independent growth, uncovering three significant genes; the Kruppel-like transcription factor, KLF4, the pro-apoptotic calcium-binding protein, PDCD6 (ALG-2) and the homeodomain pituitary transcription factor, PITX1 (which after further investigation was implicated as a negative regulator of the Ras pathway) (52).

Targeting Individual Genes *In Vitro* and *In Vivo*

Angiogenesis. Vascular endothelial growth factor (VEGF) has long been recognized as a key factor in the development and formation of novel blood vessels, and many therapeutic

strategies are specifically aimed at VEGF inhibition. It is not surprising, therefore, that RNAi silencing of VEGF is seen as an attractive opportunity to interfere with angiogenesis. siRNA was used to silence VEGF in RKO human colon cancer cells, resulting in a decrease in proliferation (53). Cationized gelatin delivery of a plasmid DNA expressing VEGF siRNA was used to silence VEGF successfully *in vitro* and *in vivo*. *In vitro* it knocked down the expression of three different VEGF isoforms in murine squamous cell carcinoma NRS-1, and *in vivo* a mouse model showed a marked reduction in vascularity accompanied by the inhibition of a VEGF siRNA transfected tumour (54).

Invasion. A characteristic feature of malignant neoplasm is invasion and metastasis. Despite advances in the management of many solid tumours, metastasis continues to be the most significant cause of cancer mortality. Many recent studies have demonstrated that RNAi is a viable approach to inhibit tumour growth and invasion/metastasis. Overexpression of RhoA or RhoC in breast cancer indicates a poor prognosis. This is due to increased tumour cell proliferation, invasion and increased tumour-dependent angiogenesis. A recent study used siRNA to silence both RhoA and B in MDA-MB-231 breast cancer cells, resulting in a decrease in proliferation and invasion. In a nude mouse model, intratumoral injections of these siRNAs almost totally inhibited the growth and angiogenesis of xenografted MDA-MB-231 tumours (55). Another study focused on the urokinase plasminogen inhibitor (uPA) and its receptor (uPAR), both of which are essential for tumour cell invasion and metastasis. Down-regulation of the inhibitor and receptor after siRNA transfection resulted in a decrease in invasion and angiogenic potential. The results also showed an increase in apoptotic cells (56). S100A4 is a protein which has only recently been associated with the promotion of invasion. A plasmid construct expressing shRNA specific to S100A4 was used to significantly reduce anaplastic thyroid tumours in nude mice. The study also showed that tumour cells were sensitized to chemotherapy as a result of S100A4 knock-down (57).

Apoptosis. The kinase Mirk/Dyrk1B was proven to mediate cell survival in pancreatic ductal adenocarcinoma through siRNA silencing. Transfection of Panc1 pancreatic cells with Mirk/Dyrk1B siRNA was sufficient to cause the induction of apoptosis (58). Raf-1, a cytosolic serine-threonine kinase, also plays an important role in apoptosis, along with tumour cell growth and proliferation. This gene has also been used as a siRNA target both *in vitro* and *in vivo*. In this study, transfections were performed on a number of cell lines including HUVEC and MDA-MB-435 and the results showed a 75% reduction in Raf-1 mRNA compared to the control groups. *In vitro* studies showed a 60% decrease in tumour growth after injection with the siRNA (59).

The above is a small example of some of the work that has been carried out using RNAi in cancer research. Thyroid carcinoma (57), bladder cancer (60), brain cancer (61), ovarian cancer (62) and pancreatic cancer (63) have all been the focus of similar studies carried out in the last 12 months.

Clinical use of RNAi. Sirna Therapeutics are currently involved in a phase 1 trial of sirna-027 as a therapy for age-related macular degeneration (AMD). Patients have been given intravitreal doses of siRNA and follow-up of up to 84 days has shown a dose-dependent improvement in sight. Importantly, the drug is safe and well-tolerated (64). The same company are also developing an antiviral RNAi against hepatitis C. The treatment has been successful in animal models and is being taken to phase 1 trials this year.

Another company, Alnylam, has developed an intranasal siRNA that is effective against respiratory syncitial virus in mice, and they are also working on siRNA-based treatment for emerging flu strains (65). In March 2006, further research by Alnylam scientists demonstrated, in primates, that a systemically delivered RNAi therapeutic can potently silence an endogenous disease-causing gene in a clinically relevant manner. Alnylam and collaborators showed silencing of the gene for apolipoprotein B (apoB), a protein involved in cholesterol metabolism, with clinically significant efficacy, as demonstrated by reductions in levels of cholesterol and low-density lipoproteins (LDL) (66). In April 2006, phase I clinical data was presented for ALN-RSV01. The drug was found to be safe and well-tolerated when administered intranasally in two phase I clinical studies. ALN-RSV01 is being evaluated for the treatment of respiratory syncytial virus (RSV) infection, and is the first RNAi therapeutic in human clinical development for an infectious disease.

Potency of RNAi. RNA interference has been developed as a viable and more effective alternative to antisense- and ribozyme-based tools for gene function and target validation studies and it has now largely displaced efforts with antisense and ribozymes.

Antisense technology suffers from various limitations, including difficulty in identifying effective sequences, low selectivity, limited *in vitro* and *in vivo* delivery and expression options, and little amenability to high-throughput screening (67, 68). While it can be difficult to directly compare the efficiency of gene suppression technologies due to differences in the rules for optimal design and target sequence selection, several studies have supported the conclusion that RNA-mediated inhibition is more potent than that achieved with antisense oligonucleotides, even in cases where site selection was optimized for antisense effectiveness (69). Also, it is easier to identify effective RNAi target sites. Although the rules for optimal RNAi effectiveness are still being

determined, when basic parameters regarding the CG content and the composition of the 3'-overhangs were met, then a high percentage of potential targets screened usually proved to be functional (70, 71). On the other hand, it is significantly more difficult to identify efficient antisense oligonucleotide target sequences, and ribozyme target selection is limited by availability of particular sequence motifs required for cleavage (67).

Stability of RNAi. Although siRNA molecules appear to be more resistant to nuclease degradation than antisense molecules (72), some serum nucleases can degrade siRNAs (73, 74). As a result, a number of groups have investigated the use of chemical modifications that improve stability and protect against nuclease degradation (75). With antisense, it has been suggested that oligonucleotides containing popular modifications such as phosphorothioate bind to proteins in the cell and can never interact with their targets (76), so that all of the effects attributed to the antisense are unrelated to the presumed targets. A randomly chosen "control" oligo may not bind in the same way to proteins and so give a false result (76).

Despite this problem, phosphothioate modification has generally been successful in antisense applications *in vivo* (77). In contrast, studies have shown that toxicity and loss of silencing activity can be a problem with phosphothioate siRNAs (78, 70). As a result, several groups have investigated the use of alternative backbone modifications to address these problems (79, 80).

Specificity of RNAi. One of the apparent advantages of RNA interference over antisense technology was that the RNAi process is highly specific. However, experimental results then suggested that siRNAs can cause off-target effects. Jackson *et al.* (81) showed that expression profiling studies revealed siRNA-specific, rather than target-specific, signatures. They concluded that siRNA could "cross-react" with other genetic targets. Since then, Persengiev *et al.* (82) have used microarrays to detect changes in well over 1000 genes following the introduction of siRNA against a target that was not even expressed in the treated cells. This study points out the uncertainty of presuming RNAi elimination of the target mRNA based on the phenotypic effect alone. Even an extensive optimization of experimental conditions, including a substantial reduction in the amounts of siRNA, did not prevent induction of a small number of genes by "control" random-sequence siRNAs (83). However, other studies have been contradictory, with researchers finding a high specificity of siRNA effects (81, 82). It has even been suggested that antisense offers a cleaner knockdown than RNAi because of reduced off-target effects.

It is interesting to note that off-target effects are not observed when complete dsRNAs are introduced instead of synthetic siRNA in primitive organisms. In *C. elegans* expression of dsRNAs of 500 base-pairs or more typically results in very efficient gene silencing, irrespective of the sequence of the target mRNA (46). One explanation for this could be that endogenously derived siRNA are generated from the cleavage of dsRNA by Dicer and RISC, which may have a proofreading mechanism that protects against the silencing of endogenous genes (46).

As with antisense, there is also the potential for siRNA to form unintended complexes with specific proteins. For example, antisense oligos targeting the Myc oncogene were found to have sequence-specific, but not antisense-specific, activities (86). This resulted in growth suppression, which, it appears, was reminiscent of, but unrelated to, the results of Myc inhibition.

It is also possible that siRNAs can act as miRNA. miRNAs do not require perfect homology to their target in order to be effective, therefore it is possible that a single siRNA can effect multiple mRNAs, resulting in off-target effects at the mRNA and protein level. Studies have shown that as little as seven to eleven consecutive homologous bases between the 5'-end of either siRNA strand to an mRNA can cause a reproducible reduction in transcript levels (86, 81).

When used *in vivo,* there is also the problem of an immune response. When long stretches of double-stranded RNA are introduced into a cell they trigger an immune response to viral infection. The introduction of shorter 21 to 23 bp siRNAs seems to overcome this problem (87). One study (88) linked the activation of interferon to the expression vectors carrying shRNA hairpins, while siRNA alone did not elicit such a response. The absence of short over-hangs, produced by the natural processing of miRNA, as well as the unconventional 5'-termini (*e.g.,* triphosphate), might explain the recognition of siRNA (expressed or transfected) as a foreign body (89). Interferon triggers the degradation of mRNA by inducing 2-5' oligoadenylate synthase, which in turn activates RNase L (90). Interferon can also activate dsRNA-dependent protein kinase (PKR), which phosphorylates eIF2. Phosphorylation of the translation initiation factor eIF2 causes its inactivation and global inhibition of mRNA activation.

A more recent study (91) showed that transfection with both a traditional and a chemically-modified siRNA containing a locked nucleic acid (LNA), into the HeLa and MCF-7 cell lines, caused no anti-viral response, whereas transfection with either long dsRNA or *in vitro*-transcribed siRNA led to a greater than 1000-fold induction of these genes.

There is also the theory that high levels of exogenous siRNA can compete with and decrease the efficiency of miRNA, as both are recognized and processed by the same cellular factors (92).

It is clear that there are several artifacts that can arise from siRNA transfection, causing a misleading result. The most common cause, however, is due to siRNA delivery. Whether *via* transfection or viral transduction, siRNA delivery can result in temporary changes in the cell and, in more extreme cases, cells may become resistant to the conditions of delivery. As mentioned above, designing siRNAs with resistance to serum RNases, without sacrificing biological activity, is possible through chemical modification. siRNAs can also be encased in cationic liposomes (93), lipid complexes (94) or collagen complexes (95). Further, they can be coupled with antibodies to cell surface receptor ligands for cell-specific delivery (96).

Given these specificity problems with RNA interference, the question arises as to which biological phenomena observed in shRNA- or siRNA-treated cells are indeed the consequences of elimination of a specific RNA. In most published reports, the biological phenomena that are observed in the siRNA-treated cells are attributed to the knockdown of the intended RNAi target, if they are not observed in the cells treated with a "control" siRNA, which is designed against a different target, or is "scrambled" to lack any recognizable target (92).

Another approach is to target the same mRNA with more than one siRNA sequence. Therefore, if both siRNAs give the same result, one can assume that the data has been interpreted adequately.

More recently, a more elaborate approach has been suggested, whereby the ultimate control for any RNAi experiment remains rescue by expression of the target gene (97). Using this method, cDNA, which is imperfectly matched to the original siRNA, is put back into the cell. Generally, redundancy in the genetic code would permit several nucleotide changes in the stretch originally targeted by the siRNA, so that the restored message could no longer be targeted. Successful reconstitution could be verified by re-appearance of the corresponding protein. Such a reconstruction procedure is expected to reverse the target-specific, but not the off-target, effects of the siRNA which remain present in the cell. However, although this method appears more cumbersome than the use of multiple siRNAs, it is more rigorous and may be considered by researchers in the future (92).

RNAi is still a technology in its early stages of development, a development which has been facilitated by the many years of research into antisense technology and the enormous amount of genetic information recently obtained from the Human Genome Project. In the space of a very short time, thousands of papers have been published in this area. However, there are still many unanswered questions, especially regarding the nature and cause of the off-target effects described above. It is expected that a higher standard of specificity may be required for publication in the future.

Acknowledgements

The authors wish to acknowledge the support of the SFI and HEA (PTRLI cycle 3 programme).

References

1 Matzke MA, Primig M, Trnovsky J and Matzke AJ: Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J *8*: 643-649, 1989.

2 Linn F, Heidmann I, Saedler H and Meyer P: Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. Mol Gen Genet *222*: 329-336, 1990.

3 Napoli C, Lemieux C and Jorgensen R: Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell *2*: 279-289, 1990.

4 Smith CJ, Watson CF, Bird CR, Ray J, Schuch W and Grierson D: Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Mol Gen Genet *224*: 477-481, 1990.

5 van der Krol AR, Mur LA, Beld M, Mol JN and Stuitje AR: Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell *2*: 291-299, 1990.

6 Wang Y, Wagner JD and Guthrie C: The DEAH-box splicing factor Prp16 unwinds RNA duplexes *in vitro*. Curr Biol *8*: 441-451, 1989.

7 Hammond SM, Bernstein E, Beach D and Hannon GJ: An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature *404*: 293-296 , 2000.

8 Kennerdell JR and Carthew RW: Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled2* act in the *wingless* pathway. Cell *95*: 1017-1026, 1998.

9 Sanchez Alvarado A and Newmark PA: Double-stranded RNA specifically disrupts gene expression during planarian regeneration. Proc Natl Acad Sci USA *96(9)*: 5049-5054, 1999.

10 Ngo H, Tschudi C, Gull K and Ullu E: Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc Natl Acad Sci USA *95*: 14687-14692, 1998.

11 Voinnet O, Vain P, Angell S and Baulcombe DC: Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell *95*: 177-187, 1998.

12 Waterhouse PM, Graham MW and Wang MB: Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci USA *95*: 13959-13964, 1998.

13 Harborth J, Elbashir SM, Bechert K, Tuschl T and Weber K: Identification of essential genes in cultured mammalian cells using small interfering RNAs. J Cell Sci *114*: 4557-4565, 2001.

14 Bernstein E, Caudy AA, Hammond SM and Hannon GJ: Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409(6818)*: 363-366, 2001.

15 Wall NR and Shi Y: Small RNA: can RNA interference be exploited for therapy? Lancet *362(9393)*: 1401-1403, 2003.

16 Hamilton A, Voinnet O, Chappell L and Baulcombe D: Two classes of short interfering RNA in RNA silencing. EMBO J *21*: 4671-4679, 2002.

17 Nicholson AW: Function, mechanism and regulation of bacterial ribonucleases. FEMS Microbiol Rev *23*: 371-390, 1999.

18 Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar PK and Mukherjee SK: RNA interference: biology, mechanism and applications. Microbiol Mol Biol Rev *67*: 657-685, 2003 (review).

19 Yang D, Buchholz F, Huang Z, Goga A, Chen CY, Brodsky FM and Bishop JM: Short RNA duplexes produced by hydrolysis with *Escherichia coli* RNaseIII mediate effective RNA interference in mammalian cells. Proc Natl Acad Sci *99(15)*: 9942-9947, 2002.

20 Blaszczyk J, Tropea JE, Bubunenko M, Routzahn KM, Waugh DS, Court DL and Ji X: Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. Structure *9*: 1225-1236, 2001.

21 Martinez J, Pathaniowska A, Urlaub H, Luhrmann R and Tuschl T: Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell *110(5)*: 563-574, 2002.

22 Novina CD and Sharp PA: The RNAi revolution. Nature *430*: 161-164, 2004.

23 Tuschl T, Zamore PD, Lehmann R, Bartel DP and Sharp PA: Targeted mRNA degradation by double-stranded RNA *in vitro*. Genes Dev *13*: 3191-3197, 1999.

24 Hamilton AJ and Baulcombe DC: A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*: 950-952, 1999.

25 Grishok A, Pasquinelli A E, Conte D, Li N, Parrish S, Ha I, Baillie D L, Fire A, Ruvkun G and Mello CC: Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell *106*: 23-34, 2001.

26 Pal-Bhadra M, Bhadra U and Birchler JA: RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. Mol Cell *9*: 315-327, 2002.

27 Mallory AC, Reinhart BJ, Jones-Rhoades MW, Tang G, Zamore PD, Barton MK and Bartel DP: MicroRNA control of Phabulosa in leaf development: importance of pairing to the microRNA 5' region. EMBO J *23*: 3356-3364, 2004.

28 Guo HS, Xie Q, Fei JF and Chua NH: MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. Plant Cell *17*: 1376-1386, 2005.

29 Doench JG, Petersen CP and Sharp PA: siRNAs can function as miRNAs. Res Commun *17(4)*: 438-442, 2003.

30 Saxena S, Jonsson ZO and Dutta A: Small RNAs with imperfect match to endogenous mRNA repress translation:implications for off-target activity of siRNA in mammalian cells. J Biol Chem *10*: 1074, 2003.

31 Zilberman D, Cao X and Jacobsen SE: *Argonaute4* control of locus-specific siRNA accumulation and DNA and histone methylation. Science *299*: 716, 2003.

32 Hammond SM: MicroRNAs as oncogenes. Curr Opin Genetics Develop *16*: 4-9, 2006.

33 Kim V: MicroRNA precursors in motion: exportin-5 mediates their export, Trends Cell Biol *4*: 156-159, 2004.

34 Lippman Z and Martienssen R: The role of RNA interference in heterochromatic silencing. Nature *431(7006)*: 364-370, 2004.

35 Lai CE: miRNAs: whys and wherefores of miRNA-mediated regulation. Curr Biol *15(12)*: R458-R460, 2005.

36 Krichevsky AM, King KS, Donahue CP, Khrapko K and Kosik KS: A miRNA array reveals extensive regulation of miRNAs during brain development. RNA *9*: 1274-1281, 2003.

37 Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M and Croce CM: Human miRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA *101*: 2999-3004, 2004.

38 Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP and James RJ: Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res *1*: 882-891, 2003.

39 Metzler M, Wilda M, Busch K, Viehmann S and Borkhardt A: High expression of precursor miRNA-155/BIC RNA in children with Burkitt lymphoma. Genes Chromosomes Cancer *2*: 167-169, 2004.

40 Pfeffer S, Zavolan M, Grasser FA, Chien M, Russo JJ, Ju J, John B, Enright AJ, Marks D, Sander C and Tuschl T: Identification of virus-encoded microRNAs. Science *304(5671)*: 734-736, 2004.

41 Li S, Crothers J, Haqq CM and Blackburn EH: Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. J Biol Chem *280(25)*: 23709-23717, 2005.

42 Caldas C and Brenton JD: Sizing up miRNAs as cancer genes. Nature Med *11*: 712-714, 2005.

43 He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ and Hammond SM: A microRNA polycistron as a potential human oncogene. Nature *435(7043)*: 828-833, 2005.

44 Behlke MA: Progress towards *in vivo* use of siRNAs. Mol Ther *4*: 644-670, 2006 [Epub February 14 2006].

45 Ryther RC, Flynt AS, Phillips III JA and Patton JG: siRNA therapeutics: big potential from small RNAs. Gene Ther *12*: 5-11, 2005.

46 Pai SI, Lin YY, Macaes B, Meneshian A, Hung CF and Wu TC: Prospects of RNA interference therapy for cancer. Gene Ther *13(6)*: 464-477, 2006.

47 Mittal V: Improving the efficiency of RNA interference in mammals. Nat Rev Genet *5(5)*: 355-365, 2004.

48 Miyagishi M and Taira K: Strategies for generation of an siRNA expression library directed against the human genome. Oligonucleotides *13(5)*: 325-333, 2003.

49 Siolas D, Lerner C, Burchard J, Ge W, Linsley PS, Paddison PJ *et al*: Synthetic shRNAs as potent RNAi triggers. Nat Biotechnol *23(2)*: 227-231, 2005.

50 MacKeigan P, Murphy LO and Blenis J: Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. Nat Cell Biol *7(6)*: 591-600, 2005.

51 Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, Lenz G, Lam LT, Dave S, Yang L, Powell J and Staudt LM: A loss-of-function RNA interference screen for molecular targets in cancer, Nature *441(7089)*: 106-110, 2006 [Epub March 29 2006].

52 Kolfschoten IG, van Leeuwen B, Berns K, Mullenders J, Beijersbergen RL, Bernards R, Voorhoeve PM and Agami R: A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. Cell *121(6)*: 849-858, 2005.

53 Mulkeen AL, Silva T, Yoo PS, Schmitz JC, Uchio E and Cha C: Short interfering RNA-mediated gene silencing of vascular endothelial growth factor: effects on cellular proliferation in colon cancer cells. Arch Surg *141(4)*: 367-374, 2006.

54 Matsumoto G, Kushibiki T, Kinoshita Y, Lee U, Omi Y, Kubota E and Tabata Y: Cationized gelatin delivery of a plasmid DNA expressing small interference RNA for VEGF inhibits murine squamous cell carcinoma. Cancer Sci *97(4)*: 313-321, 2006.

55 Pillé JY, Denoyelle C, Varet J, Bertrand JR, Soria J, Opolon P, Lu H, Pritchard LL, Vannier JP, Malvy C Soria C and Li H: Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells *in vitro* and *in vivo*. Mol Ther *11*: 267-274, 2005.

56 Subramanian R, Gondi CS, Lakka SS, Jutla A and Rao JS: siRNA-mediated simultaneous downregulation of uPA and its receptor inhibits angiogenesis and invasiveness triggering apoptosis in breast cancer cells. Int J Oncol *28(4)*: 831-839, 2006.

57 Shi Y, Zou M, Collison K, Baitei EY, Al-Makhalafi Z, Farid NR and Al-Mohanna FA: RNA interference targeting S100A4 (Mts1) suppresses tumor growth and metastasis of anaplastic thyroid carcinoma in a mouse model. J Clin Endocrin Metab, 2006.

58 Deng X, Ewton DZ, Li S, Naqvi A, Mercer SE, Landas S and Friedman E: The kinase Mirk/Dyrk1B mediates cell survival in pancreatic ductal adenocarcinoma. Cancer Res *66(8)*: 4149-4158, 2006.

59 Leng Q and Mixson AJ: Small interfering RNA targeting Raf-1 inhibits tumor growth *in vitro* and *in vivo*. Cancer Gene Ther *12*: 682-690, 2005.

60 Nogawa M, Yuasa T, Kimura S, Tanaka M, Kuroda J, Sato K, Yokota A, Segawa H, Toda Y, Kageyama S, Yoshiki T, Okada Y and Maekawa T: intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer. J Clin Invest *115*: 978-985, 2005.

61 Boado RJ: RNA interference and nonviral targeted gene therapy of experimental brain cancer. J Am Soc Exper Neuro Therapeut *2*: 139-150, 2005.

62 Noske A and Pahl S: Combined adenosquamous and large-cell neuroendocrine carcinoma of the gallbladder. Virchows Arch. Mar 29, 2006 [Epub ahead of print].

63 Bhattacharyya M and Lemoine NR: Gene therapy developments for pancreatic cancer. Best Pract Res Clin Gastroenterol *20(2)*: 285-298, 2006.

64 Quinlan E: ARVO Annual Meeting, 1-5 May, Ft. Lauderdale, FL, USA, 2005.

65 Bitko V, Musiyenko A, Shulyayeva O and Barik S: Inhibition of respiratory viruses by nasally administered siRNA. Nat Med *11(1)*: 50-55, 2005 [Epub December 26 2004].

66 Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, Harborth J, Heyes JA, Jeffs LB, John M, Judge AD, Lam K, McClintock K, Nechev LV, Palmer LR, Racie T, Rohl I, Seiffert S, Shanmugam S, Sood V, Soutschek J, Toudjarska I, Wheat AJ, Yaworski E, Zedalis W, Koteliansky V, Manoharan M, Vornlocher HP and MacLachlan I: RNAi mediated gene silencing in non-human primates. Nature *441(7089)*: 111-114, 2006 [Epub March 26 2006].

67 Scherer LJ and Rossi JJ: Approaches for the sequence-specific knockdown of mRNA. Nature Biotechnol *12*: 1457-1465, 2003.

68 Dias N and Stein CA: Antisense oligonucleotides: basic concepts and mechanisms. Mol Cancer Ther *1*: 347-355, 2002.

69 Uprichard S: The therapeutic potential of RNA interference. FEBS Lett *579*: 5996-6007, 2005.

70 Harborth J, Elbashir S, Vandenburgh K, Manninga H, Scaringe S, Weber K and Tuschl T: Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev *13(2)*: 83-105, 2003.

71 Reynolds A, Leake D, Boese Q, Scaringe S, Marshall W and Khvorova A: Rational siRNA design for RNA interference. Nat Biotechnol *22(3)*: 326-330, 2004.

72 Bertrand JR, Pottier M, Vekris A, Opolon P, Makdimenko A and Malvy C: Comparison of antisense oligonucleotides and siRNAs in cell culture and *in vivo*. Biochem Biophys Res Commun *296(4)*: 1000-1004, 2002.

73 Paroo Z and Corey DR: Challenges for RNAi *in vivo*. Trends Biotechnol *22(8)*: 390-394, 2004.

74 Dykxhoorn DM and Lieberman J: The silent revolution: RNA interference as basic biology, research tool, and therapeutic. Annu Rev Med *56*: 401-423, 2005.

75 Fougerolles A, Manoharan M, Meyers R and Vornlocher HP: RNA interference *in vivo*: toward synthetic small inhibitory RNA-based therapeutics. Methods Enzymol *392*: 278-296, 2005.

76 Bruker L and Tremblay GA: Cellular proteins prevent antisense phosphorothioate oligonucleotide (SdT18) to target sense RNA (rA18): development of a new *in vitro* assay. Biochemistry *39(37)*: 11463, 2000.

77 Crooke ST: Progress in antisense technology. Ann Rev Med *55*: 61-95, 2004.

78 Braasch DA, Jensen S, Liu Y, Kaur K, Arar K, White MA *et al*: RNA interference in mammalian cells by chemically-modified RNA. Biochemistry *42(26)*: 7967-7975, 2003.

79 Elmen J, Thonberg H, Ljungberg K, Frieden M, Westergaard M and Xu Y *et al*: Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. Nucleic Acids Res *33(1)*: 439-447, 2005.

80 Allerson CR, Sioufi N, Jarres R, Prakash TP, Naik N, Berdeja A *et al*: Fully 2'-modified oligonucleotide duplexes with improved *in vitro* potency and stability compared to unmodified small interfering RNA. J Med Chem *48(4)*: 901-904, 2005.

81 Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Cavet G and Linsley PS: Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol *21(6)*: 635-637, 2003.

82 Persengiev SP, Zhu X and Green MR: Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA *10(1)*: 12-18, 2004.

83 Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN and Fesik SW: Specificity of short interfering RNA determined through gene expression signatures. Proc Natl Acad Sci USA *100(11)*: 6347-6352, 2003.

84 Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE and Mello CC: Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*: 6669, 806-811, 1998.

85 Chavany C, Connell Y and Neckers L: Contribution of sequence and phosphorothioate content to inhibition of cell growth and adhesion caused by c-myc antisense oligomers. Mol Pharmacol *48(4)*: 738-746, 1995.

86 Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, Lee JC, Hughes CM, Shanmugam KS, Bhattacharjee A, Meyerson M and Collins FS: Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. Proc Natl Acad Sci USA *101(7)*: 1892-1897, 2004.

87 Paddison PJ, Caudy AA and Hannon GJ: Stable suppression of gene expression by RNAi in mammalian cells. PNAS *99(3)*: 1443-1448, 2002.

88 Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL and Iggo R: Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet *34(3)*: 263-264, 2003.

89 Marques JT and Williams BR: Activation of the mammalian immune system by siRNAs. Nat Biotechnol *23(11)*: 1399-1405, 2005.

90 Stark GR, Kerr IM, Williams BR, Silverman RH and Schreiber RD: How cells respond to interferons. Annu Rev Biochem *67*: 227-264, 1998.

91 Dahlgren C, Wahlestedt C and Thonberg H: No induction of anti-viral responses in human cell lines HeLa and MCF-7 when transfecting with siRNA or siLNA. Biochem Biophys Res Commun *341(4)*: 1211-1217, 2006 [Epub January 30 2006].

92 Gartel AL and Kandel ES: RNA interference in cancer. Biomol Eng *23(1)*: 17-34, 2006 [Epub February 8, 2006] (Review).

93 Matsumoto G, Kushibiki T, Kinoshita Y, Lee U, Omi Y, Kubota E and Tabata Y: Cationized gelatin delivery of a plasmid DNA expressing small interference RNA for VEGF inhibits murine squamous cell carcinoma. Cancer Sci *97(4)*: 313-321, 2006.

94 Santel A, Aleku M, Keil O, Endruschat J, Esche V, Durieux B, Loffler K, Fechtner M, Rohl T, Fisch G, Dames S, Arnold W, Giese K, Klippel A and Kaufmann J: RNA interference in the mouse vascular endothelium by systemic administration of siRNA-lipoplexes for cancer therapy.Gene Ther Apr 20, 2006 [Epub ahead of print].

95 Minakuchi Y, Takeshita F, Kosaka N, Sasaki H, Yamamoto Y, Kouno M, Honma K, Nagahara S, Hanai K, Sano A, Kato T, Terada M and Ochiya T: Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing *in vitro* and *in vivo*. Nucleic Acids Res *32(13)*: e109, 2004.

96 Schiffelers RM, Mixson AJ, Ansari AM, Fens MH, Tang Q, Zhou Q, Xu J, Molema G, Lu PY, Scaria PV, Storm G and Woodle MC: Transporting silence: design of carriers for siRNA to angiogenic endothelium, J Control Release *109(1-3)*: 5-14, 2005 [Epub June 24 2005].

97 Editorial [no authors listed]: Whither RNAi. Nature Cell Biol *5(6)*: 489-490, 2003.

Received May 31, 2006
Accepted June 6, 2006