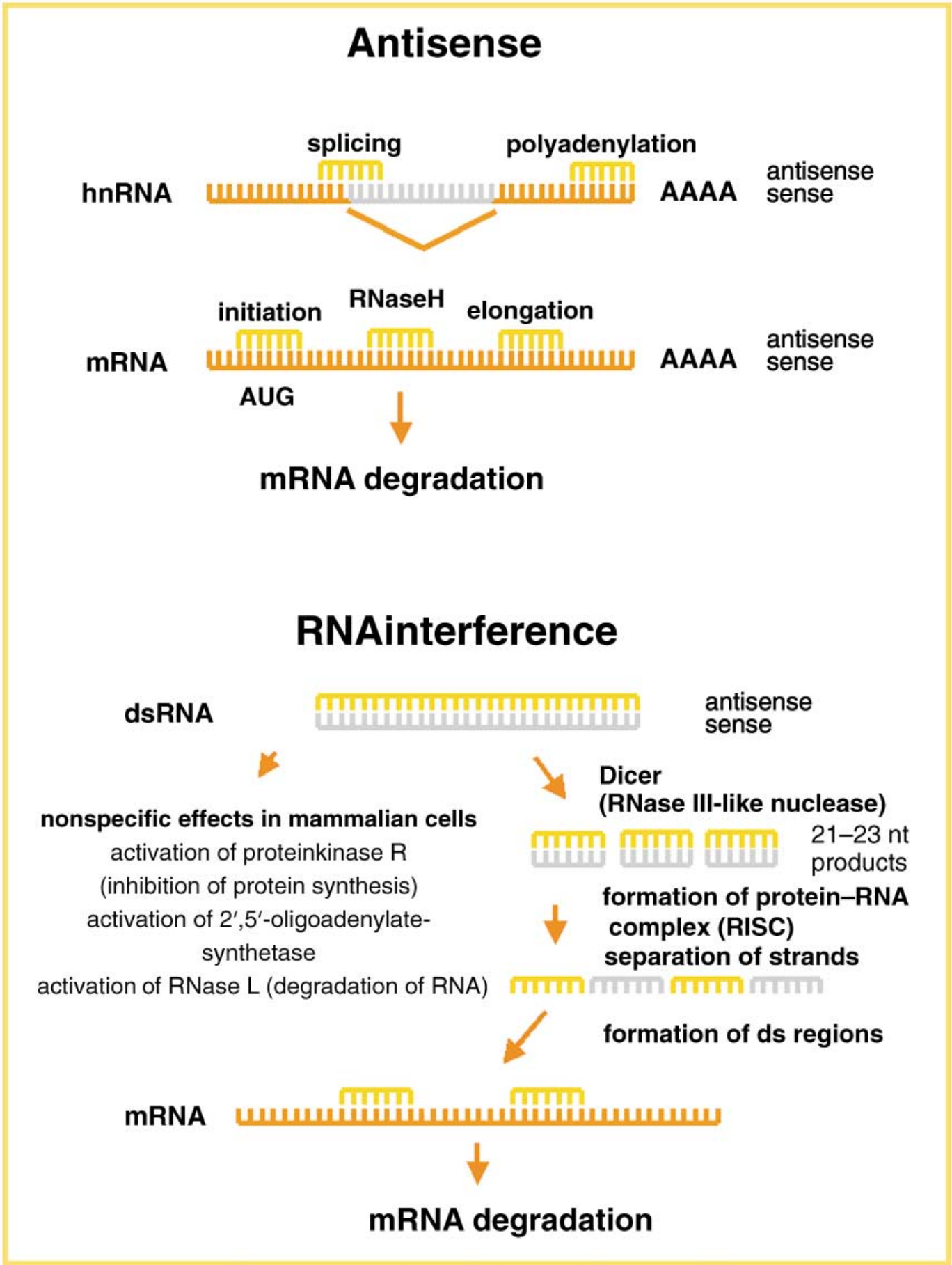

  DOI: 10.1002/cbic.200300708

Oligonucleotide-Based Knockdown Technologies: Antisense Versus RNA Interference

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The postgenomic era is characterized by an almost intimidating amount of information regarding the sequences and expression of previously unknown genes. In response, researchers have developed an increasing interest in functional studies. At the start of such a study, one may have little more than sequence information and bioinformatic annotation. The next step is to hypothesize a potential role in the context of a cell. Testing of the hypothesis needs to be fast, cheap, and applicable to a large number of genes. Knockdown methods that rely on binding of antisense oligonucleotides to mRNA combined with a subsequent functional assay in cell culture fulfil these requirements: sequence information is sufficient for synthesis of active inhibitors. Depending on the in vitro model chosen, knockdown of gene expression can be achieved with medium or even high throughput. The two most popular methods of knockdown in cell culture are the use of antisense oligonucleotides that rely on ribonuclease H (RNAse H)-dependent cleavage of mRNA, and RNA interference triggered by small double-stranded RNA molecules. Both methods act in a sequence-specific manner and can give efficient knockdown. In both cases, researchers struggle with nonspecific "off-target" effects and the difficulty of site selection. Studies that compare the methods differ in their judgment as to which method is superior.

Introduction

Methods of knockdown

Inhibition of expression by nucleic acids has been known to occur for more than two decades. In 1978 Stephenson and Zamecnik utilized sequence-specific binding of oligonucleotides to interfere with the translational machinery.[1] Ever since, researchers have used antisense nucleic acids to manipulate gene expression and thereby identify gene functions. Among the different techniques that have emerged are several that use a changed backbone of the antisense oligodeoxynucleotide (As-ODN) to improve serum stability or hybridization with the target mRNA.[2–5] As-ODNs can be used specifically to interfere with splicing and cause one splice variant to be favored over another.[6, 7] There is one example of redirection of polyadenylation by As-ODN.[8] However, most applications of As-ODN aim at the knockdown of a target molecule, either by translational blocking or the activation of ribonuclease H (RNase H).

An alternative to synthetic antisense oligonucleotides is intracellularly expressed antisense RNA. Expressed RNA has proven very successful, particularly in its in vivo application.[9] In 1998 the groups of Fire and Mello discovered RNA interference (RNAi), the inhibition of gene expression by double-stranded RNA (dsRNA).[10] While this new and powerful technique worked in a sequence-specific manner in model organisms such as *Caenohabditis elegans* and *Drosophila*, a nonspecific response was triggered in mammalian cells.[11] Only the finding that this interferon response can be circumvented by the usage of short interfering RNAs (siRNAs) of 21 nucleotides (nt) made it possible to apply RNAi in mammalian cells.[12]

While both classic antisense approaches and the use of RNAi rely on the presence of an executing nuclease in the cell, ribozymes (recently discussed by Rossi)[13] combine sequence-specific homologous binding with the capability of RNA to act as an enzyme that directly cuts the target mRNA.

Binding of RNA or DNA aptamers is not based on sequence homology. Aptamers form three-dimensional structures that recognize three-dimensional epitopes. The sequence of a high-affinity binder cannot be predicted at present and is determined by an in vitro selection process.[14, 15] As a result of the adaptive recognition process of aptamers, target molecules are not restricted to mRNA. In fact the mode of action of aptamers resembles that of antibodies—yet another tool for cellular knockdown. Neutralizing antibodies can be effective at inactivating secreted proteins without further enhancement; for intracellular applications, chromophore-assisted laser inactivation has proven useful.[16]

There are a number of different techniques that can be used to knock down a gene product. The method of choice will depend on the specific purpose; duration of the experiment, stability and localization of the protein, accessibility of the cell, or species of the organism all have an effect. In the following discussion, we will focus on a comparison of As-ODNs acting

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 DOI: 10.1002/cbic.200300708

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through RNase H with siRNAs—methods of knockdown that are widely used for functional studies of gene products in mammalian cell cultures. The mechanisms of action of As-ODNs and siRNAs show similarities: they bind to target RNA in a sequence-specific manner and can activate nucleases that cause RNA degradation. In the nature of these executing nucleases, however, the two types of knockdown differ.

Antisense

Among the antisense mechanisms of oligonucleotides, RNase H-dependent cleavage of mRNA is used most often and results in the most effective knockdown.[17] To utilize RNase H, As-ODNs are designed that span one of the known and relatively frequently occurring RNase H cleavage sites. RNase H is present in most cells and is therefore a reliable executing nuclease. As a result of its nuclear localization, RNase H can act on immature and mature mRNA.

RNAi

RNAi is a cellular mechanism of defence (reviewed by Zamore):[18] recognition and cleavage of dsRNA inhibits infection by, and replication of RNA viruses, as well as hopping of transposable elements. In the first step, dsRNA is cleaved into 21-nt RNA fragments. In the second step the fragments trigger the hydrolysis of RNA at sites of close sequence identity. The components of the enzyme complex RISC that catalyzes the first cleavage of dsRNA have been partly identified; Dicer is the active nuclease in this first step.[19] The nuclease that catalyzes the second step, the digestion of RNA triggered by 21-nt fragments, is still unknown. RNAi is well conserved across eukaryotes and can therefore be utilized as a tool for knockdown in many cells and organisms. While in *C. elegans* and *Drosophila* long dsRNA is used to trigger the cleavage of mRNA identical in sequence, in mammalian cells siRNA is used. siRNA is composed of a 21-nt dsRNA with a 19-base-pair double helix and 2-nt 3′-overhangs on both strands, like Dicer cleavage fragments.[20, 12] siRNA that is slightly longer or shorter than 21 nt is less inhibitory. The presence of 2-nt 3′-overhangs is also critical. siRNA without any or with different overhangs (longer, shorter, or 5′-overhangs) is less effective.[21]

Comparison of the Antisense and RNAi Approaches

Specificity

Selective and specific inhibition of gene expression with antisense technology was improved by introducing various changes into ODN backbone chemistry (a comprehensive analysis of this topic has been presented by Freier and Altmann[22]). Phosphorothioates increase the serum stability of ODNs while retaining their ability to activate RNase H. Phosphorothioates, however, are toxic. ODNs with alkylated ribose derivates, including the often-used 2′-*O*-(2-methoxyethyl (or methyl)) ribonucleosides (MOE), show enhanced hybridization affinity but are not recognized by RNase H. Chimeras, which contain phosphorothioates on selected nucleotides in the centre of an ODN sequence flanked by MOE, are now considered to be the best reagents for use in mammalian cell culture because, compared to other As-ODNs, they are more stable and have increased affinity to the target mRNA, and they also activate RNase H.[23]

There are still unspecific effects of chimeric As-ODNs. One problem is toxicity: As-ODNs cause concentration- and cell-type-dependent cell death. The other problem is non-sequence-specific binding to proteins. As-ODNs have been shown to bind to a number of different proteins and to thereby cause significant nonspecific effects.[24, 25]

To trigger RNAi, researchers use unmodified dsRNA. In mammalian cells the utility of RNAi had been limited by the innate immune response triggered by dsRNA. Long dsRNAs induce the interferon response, which leads to the inhibition of protein translation through the protein kinase R pathway and activation of ribonuclease L. The interferon response results in a general inhibition of gene expression and significantly alters the cell physiology. This response can be avoided in mammalian cell culture by using synthetic siRNAs with a length of 21 nt.[11] However, while RNA smaller than 35 nt is too short to induce

the interferon response,[26, 27] the specificity of the gene silencing induced by siRNAs in mammalian cells has not been systematically examined. Various groups have recently published data obtained by using Affymetrix chip technology for a genome-wide expression analysis of cultured cells treated with siRNA. The studies were aimed at characterizing the specificity of the silencing effect.

The analysis Jackson and co-workers[28] performed in HeLa cells revealed only a few genes regulated together by different siRNAs targeted at the same gene. Transcript profiles showed siRNA-specific rather than target-specific signatures. A contiguous stretch of as few as eleven nucleotides that matched the siRNA sufficed to induce silencing of nontargeted genes. The introduction of mismatches into the siRNA abolished not only down-regulation of the target gene but down-regulation of nontargeted genes as well. Both the antisense and the sense strand of the siRNA directed inhibition of nontargeted genes even at very low doses. These results demonstrate that siRNAs can cross-react with genes of limited sequence similarity and therefore caution is needed in the utilization of RNAi.

In contrast, Semizarov et al.[29] presented data indicating that siRNA is a highly specific tool for targeted gene knockdown. They designed several siRNAs against different regions of three target genes. The authors had observed nonspecific induction of a set of common genes when using siRNA at a concentration of 100 nM. Genes including apoptosis-related genes (biological response modified, activated killer, B cell lymphoma 2, death-associated protein kinase) and stress-response genes (growth arrest and DNA damage, p38, mitogen-activated protein kinase) were up-regulated in the cells in a concentration-dependent manner. To avoid these nonspecific effects, the authors first optimized their silencing experiments. For microarray analysis they eventually used siRNA at 5 – 20 nM concentrations. Under optimized transfection conditions the signatures for different siRNAs against the same target gene were shown to correlate very closely, whereas the signatures for different targets revealed no correlation. The authors conclude that siRNA can be a highly specific tool for targeted gene knockdown, which establishes siRNA-mediated gene silencing as a reliable approach for large-scale screening of gene function and drug target validation.

Similar results were presented by Chi et al.[30] They used human embryonic kidney (HEK 293) cells that express green fluorescent protein (GFP) and siRNAs directed against GFP. Our own experimental data are in accordance with the findings of the groups of Semizarov and Chi. With 1 – 1000 nM siRNA in adipocytes we observed sequence-specific knockdown at low concentrations in a genome-wide expression analysis. At high concentrations, nonspecific effects were noticeable (Figure 1).

In each cellular system the concentration of siRNAs, the transfection reagent, and the combination of the two have to be optimized before the actual gene silencing experiment. More generally, both knockdown technologies, antisense oligonucleotides and RNAi, can be used to elucidate gene functions, to identify drug targets, or to develop more specific therapeutics. However, one has to keep in mind that the specificity of the technology has to be proven for each specific knockdown tool.

Level of knockdown

Comparing the level of knockdown achieved with RNase H-dependent antisense and RNAi approaches is a challenging task since the design rules for sequence and site selection, as well as optimal transfection conditions are different. Researchers from Novartis Pharma[31, 32] exploited the cellular system of the rat pain-related cation channel $P2X_3$ expressed in chinese hamster ovary (CHO-K1) cells and a rat brain tumor-derived cell line, 33B. They started by designing As-ODNs against the target sequence. For the sites that worked best for the As-ODN approach, they then generated siRNAs. To compare the two methods, they chose transfection conditions that worked well for both As-ODNs and siRNAs. Even though the site was selected for optimal antisense effect, siRNAs showed better inhibition of mRNA at lower doses. In a functional assay, both technologies provided successful interference with channel activity. However, approximately 10-fold higher concentrations of As-ODN were needed to achieve inhibition comparable to that achieved by siRNA.

In a similar approach to comparing As-ODN and siRNA, Vickers et al.[33] designed siRNAs for sites that allow RNase H-dependent antisense activity against the target gene phosphatase and tensin homologue deleted on chromosome 10 (PTEN). In accordance with the findings described above, siRNAs performed well against sites that allow RNase H-dependent antisense action, although not better than As-ODNs.

Use of the reverse approach, designing As-ODNs against a site that is ideal for siRNA, has only been published for one example using the siRNA against firefly luciferase originally published by Elbashir et al.[20] As one might expect, an antisense oligonucleotide against a gene with the same sequence is active but inferior to siRNA in this case.[34]

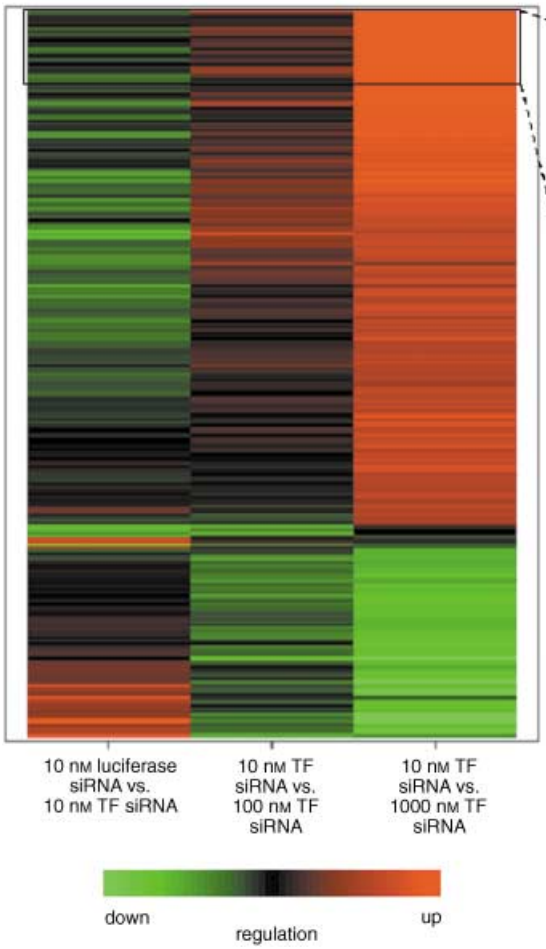
Hit rate/site selection

There are no generally accepted, standardized knockdown criteria. Successful knockdown can mean anything between 50 and 100% reduction of mRNA concentration, protein expression, or enzyme activity. To deal with this problem one needs to carefully look at the experimental data when comparing the hit rates found in studies performed by different groups.

Vickers et al.[33] designed 40 siRNAs and 40 2′-MOEs for intracellular adhesion molecule-1 (ICAM-1) mRNA. The target sequence included the 5′-untranslated region (UTR), the coding sequence, and the 3′-UTR. At identical concentrations, 30% of the As-ODNs were active but only 23% of the siRNAs. Successful knockdown was defined as 50% or greater reduction of mRNA concentration. Similar results were presented for PTEN, for which 61% of the As-ODNs and 33% of the siRNA constructs were active.

When interpreting these data one has to keep in mind that the design was more suitable for As-ODNs than for siRNA. The design rules for siRNA published by the Tuschl group were not followed. In most cases the GC content was too high for siRNAs (more than 60%) and no complementary AA residues were present in the mRNA opposite the dTdT additions in the

A



B

Affymetrix qualifier	UniGene symbol	NetAffx description
101317_F_AT	Ifnab	interferon alpha family, gene B
101788_F_AT	Ifna1	interferon alpha family, gene 1
101789_I_AT	Ifna4	interferon alpha family, gene 4
101790_F_AT	Ifna4	interferon alpha family, gene 4
101791_F_AT	Ifna5	interferon alpha family, gene 5
102148_F_AT	Ifna9	interferon alpha family, gene 9
102149_F_AT	Ifna7	interferon alpha family, gene 7
102150_F_AT	Ifna5	interferon alpha family, gene 5
102218_AT	Il6	interleukin 6
104156_R_AT	Atf3	activating transcription factor 3
112696_AT	4732465I17Rik	RIKEN cDNA 4732465I17 gene
112908_AT	Axud1	AXIN1 up-regulated 1
116877_AT	EST cluster	highly similar to A39748 protein VGF
131389_AT	EST cluster	clone:E230002F20; product:hypothetical protein
137913_AT	4833411O04Rik	RIKEN cDNA 4833411O04 gene
160463_AT	Myd116	myeloid differentiation response gene 116
160909_AT	Sprr1a	small proline-rich protein 1A
161115_R_AT	Spi13	serine protease inhibitor 13
163984_AT	Maff	musculoaponeurotic fibrosarcoma oncogene F
94145_AT	Ifnb	interferon beta, fibroblast
94146_AT	Ccl4	chemokine (C-C motif) ligand 4
94717_F_AT	Ifna2	interferon alpha family, gene 2
96419_F_AT	Ifna6	interferon alpha family, gene 6
98406_AT	Ccl5	chemokine (C-C motif) ligand 5
99392_AT	Tnfaip3	tumor necrosis factor, alpha-induced protein 3

Figure 1. *Gene expression analysis of mouse 3T3-L1 adipocytes treated with luciferase siRNA and transcription factor (TF) siRNA. Mature 3T3-L1 adipocytes were seeded in six-well plates at a density of 10^6 cells per well. Cells were treated for 24 h with 10, 100, and 1000 nM siRNA directed against luciferase and TF, respectively, in duplicate experiments. For Affymetrix GeneChip analysis, 5 μg total RNA was amplified by in vitro transcription and hybridized to the murine MG-U74ABCv2 chip series, which represents 36899 gene probe sets. Merging of signal intensities from duplicate experiments was followed by fold-change calculations: 10 nM luciferase versus 10 nM TF, 10 nM TF versus 100 nM TF, and 10 nM TF versus 1000 nM TF. The top 244 regulated genes were identified as those for which the filter criteria $p < 0.02$ and fold change > 2 applied to one or more of the three comparisons. A) Self-organizing map (SOM) clustering of ln-transformed fold-change values of the top 244 regulated genes. Red indicates relative up- and green relative down-regulation. Each row represents one gene probe set. Note the low overall gene regulation in the comparisons 10 nM luciferase versus 10 nM TF and 10 nM TF versus 100 nM TF. Unspecific siRNA effects become apparent with the 1000 nM TF siRNA. B) The list shows the top 25 up-regulated genes of the 10 nM TF versus 1000 nM TF comparison. The large number of interferon and chemokine genes indicates response to stress.*

antisense strand. In addition, the transfection reagent lipofectin had been optimized for As-ODNs. These facts may explain why just three efficient siRNA constructs were found that down-regulate mRNA levels to less than 20% at a 100 nM concentration. Considering that the siRNA molecules used in the study were not optimally designed, the inhibitory activity of siRNA targeted against open RNase H sites is remarkable. The authors postulate shared regions of greater activity ('hot spots') along the transcript for siRNAs and RNAse H-dependent As-ODNs.

In other publications the hit rate of siRNAs tends to be higher than that for As-ODNs. In a study by Hemmings-Mieszczak et al.[31] 20% of the As-ODNs tested against $P2X_3$ were found to be inhibitory. The authors subsequently designed siRNA against the sites that had worked for As-ODN: all siRNAs were inhibitory.

As a result of the difficulties posed by the different sequence requirements for the As-ODN and siRNA approaches, a direct comparison is not easily made. Our best source of information may be the accumulating published results of silencing experiments using either optimized As-ODNs or optimized siRNAs. In general researchers using RNAi report higher hit rates than researchers using antisense oligonucleotides.[31, 35] In our own hands, hit rates for As-ODNs are around 10 – 20%, and for siRNAs around 20 – 40%, where a greater than 70% reduction of mRNA concentration was considered a successful knockdown.

Bohula et al. suppressed insulin-like growth factor 1 receptor (IGF1R) expression by using a published As-ODN.[36, 37, 36, 37] Since they did not get sufficient knockdown, they started to identify alternative sites for molecular targeting.[38] By using an array-based screen they identified sites within the human IGF1R transcript that were accessible to RNAse H-mediated cleavage. Structural constraints were shown to govern the activities of As-ODNs and also those of siRNAs. Their success rate for effective

siRNA design was clearly higher than that of approximately 10% reported for As-ODNs.[39, 38] Moreover, siRNAs caused greater inhibition of expression and induced more profound dose-dependent reduction in IGF1R protein levels.

Duration of effect

Vickers et al. found similar duration of action for As-ODNS and siRNAs against B cell lymphoma x on the mRNA level.[33] The inhibition was found to be maximal 24 h post transfection and returned to normal levels by Day 5 for siRNA. The results are similar for As-ODNs except that the maximal activity was achieved 8 h after transfection.

Direct comparison of the best-working As-ODN and the best-working siRNA for $P2X_3$ showed a transient inhibition of the target with siRNAs capable of inhibition for up to 72 h, and As-ODNs just 24 h.[31] In our own experience, siRNAs can achieve complete knockdown for 96 h in luciferase assays (100 nM partially phosphorothioated and unmodified siRNA directed against firefly luciferase; see Figure 2). As-ODNs have been reported to down-regulate protein levels 48 h after transfection.[40]

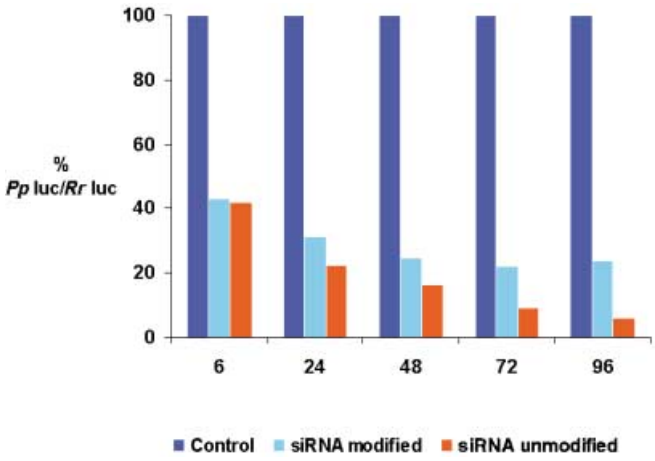


Figure 2. *Determination of the duration of the RNAi effect in HEK 293 cells. Inhibition of firefly luciferase (*Photinus pyralis (Pp) *luc) by 100 nM siRNA was measured using the dual luciferase (*Pp *luc/*Renilla reniformis (Rr) *luc) system (Promega). Modified oligonucleotides were partially phosphorothioated.*

By using six different siRNA constructs targeting the hepatitis C virus (HCV) replicon in Huh-7 cells, Wilson et al. found two siRNAs that elicit efficient inhibition: HCV nonstructural protein levels were below the detection limit of western blot analysis.[41] RNAi protected cells from HCV replicon RNA replication. To investigate the duration of RNAi of the HCV replicon, the authors first introduced siRNA to induce RNAi and then retransfected the cells with HCV replicon RNA at various times. When the replicon was transfected 96 h and 120 h after induction of RNAi the effect had become weaker. As has been shown in other mammalian systems, the effect of RNAi mediated by exogenously added synthetic siRNAs is short-lived and seems to last for 96 h. To prolong the duration of RNAi, Wilson and co-workers used a bicistronic plasmid expressing the complementary siRNAs. The protective interference activity could then be extended beyond three weeks.

Another comparison of methods was made by down-regulating IGF1R to enhance radiosensitivity and cytotoxicity of cells. Sun et al.[36, 37] used an As-ODN to suppress IGF1R in T24 cells. The inhibition by IGF1R antisense ODN was dose and time dependent. Significant inhibition occurred 12 – 24 h after application of IGF1R As-ODN. The group examined enhanced drug sensitivity on T24 cells treated with IGF1R As-ODN for 72 h. Other researchers started work on plasmid-driven IGF1R antisense experiments to increase the duration of knockdown.[36]

To summarize, both methods, RNAi and antisense knockdown, are transient and only active for 24 – 72 h, depending on the secondary readout. To prolong the down-regulation, a plasmid-driven vector can be used for all knockdown methods.

Efficacy

As-ODNs and RNAi appear to be powerful tools for functional studies of genes with unknown function. However, in both technologies, oligonucleotides targeted at different sites within a gene differ in their efficacy. Potentially, the efficacy depends as much on the secondary structure of mRNA and oligonucleotide, the accessibility of the site, and the affinity of the oligonucleotide for the target mRNA as on the tool one is using.

In agreement with this hypothesis, Vickers et al.[33] found a significant degree of correlation between active RNase H-dependent As-ODNs and siRNAs. The authors suggest that if a site is available for hybridization to an RNase H-dependent As-ODN, then it is also available for hybridization and cleavage by the siRNA complex.

On the other hand, Hemmings-Mieszczak and co-workers, who also compared As-ODNs and siRNAs with overlapping sequences, postulate that an RNAi-based method for targeting gene expression is less sensitive to the local restraints of the mRNA structure than is the antisense approach. This opinion is supported by others.[12, 31]

Miyagishi et al.[35] designed siRNAs and As-ODNs targeted at six different sites in a firefly gene. They used a luciferase reporter in an optimized system (for both the As-ODNs and the siRNAs) and found one As-ODN and two siRNAs that reduce luciferase activity to less than 10% of control levels. The active concentrations of the reagents were quite different though: the IC_{50} values for the siRNAs were about 100-fold lower than those for the As-ODNs. Once again the target site for the best-working As-ODN also worked for one of the siRNA molecules.

Efficiency of cellular uptake

The accessibility of cells is an issue for both As-ODNs and siRNAs; transfection conditions have to be optimized for every cell type. The requirements for distribution within the cell are different: while As-ODNs act in the nucleus (the compartment of RNase H activity), siRNAs most likely act in the cytoplasm, where one finds RISC.

The accessibility of a cell is dependent on the proliferation status. RNAi and As-ODNs seem to be most effective in cells in the exponential growth phase.[31] Once cells are differentiated and their proliferation capacity is low (for example, adipocytes, chondrocytes, neuronal cells), transfecting them with As-ODNs or siRNAs is a challenge.

In most cases the combination of cell type and transfection reagent is critical. Hemmings-Mieszczak and co-workers found that siRNAs show good transfection rates with oligofectamine, while As-ODNs are best transfected by using lipofectin. Transfection of siRNAs did not work with lipofectin and nor did oligofectamine for As-ODNs. To be able to compare both methods, Hemmings-Mieszczak and co-workers used electroporation, a delivery method that works for all kinds of oligonucleotides. In different cells, Vickers et al.[33] successfully used lipofectin for both As-ODNs and siRNA molecules.

Toxicity

Miyagishi et al.[35] performed cotransfection experiments by using firefly and *Renilla* luciferase. When they used As-ODNs at a concentration of 200 nM or higher, nonspecific effects occurred that blocked protein synthesis; reporter assays gave poor results because of low overall luciferase activity (*Renilla* luciferase was used as a control). They saw these nonspecific effects with a number of different transfection reagents. Their findings are consistent with our own data, which show toxicity for As-ODNs and siRNAs carrying phosphorothioates. In contrast, unmodified siRNA molecules cause no detectable inhibition of overall luciferase activity (data not shown). In a more sensitive assay (oligo chip analysis), unmodified siRNAs showed toxic side effects only above a concentration of 100 nM (Figure 1). This concentration threshold differs from the results published by Semizarov et al.[29] Most likely toxic concentrations vary with cell type and transfection conditions. To eliminate any side effects every combination of cell type, transfection reagent, and As-ODNs or siRNAs has to be optimized.

Application in vivo

Use of transient gene knockdown in vivo as a therapy, with As-ODNs or siRNAs as drugs, is the ultimate application of the technology that many researchers hope for.

Delivery of RNAse H As-ODNs to a variety of target tissues by parenteral and nonparenteral routes of administration with subsequent inhibition of gene expression has been well documented in rodents, nonhuman primates, and humans.[42–44] Butler and co-workers showed that the down-regulation of PTEN very specifically reverses hyperglycemia in diabetic mice. Administering Bcl-2 As-ODNs to patients with non-Hodgkin lymphoma led to a down-regulation of Bcl-2 and to reduction in tumor size in some patients.[44]

Other publications have documented nonspecific toxicity at high doses in rats.[45, 46] Schobitz et al. showed that both fully and partially phosphorothioated ODNs dose-dependently elevate body temperature, suppress food and fluid intake, and inhibit night-time activity. Apparently these effects do not depend on the nucleotide sequence as missense ODNs produced comparable changes in the behavioral parameters. These findings clearly indicate that centrally administered ODNs produce transient and sequence-independent effects as a result of their nucleic acid structure, in addition to their intended sequence-specific effects.

Preliminary reports of siRNA molecules delivered to mice have been published by Lewis et al.[47] The authors administered the siRNAs by rapid tail-vein injection of a large volume of fluid (high pressure delivery) and it is not clear whether administration of siRNAs by more clinically acceptable practices will result in effective delivery to target tissues.

McCaffrey and co-workers[45] have presented similar results. They used a modification of the hydrodynamic high-pressure transfection method to deliver naked siRNAs and a luciferase reporter plasmid to the liver. Specific siRNA-mediated inhibition of luciferase expression was observed in adult mice. Although these results show that siRNAs are functional in mice without chemical modification, delivery remains a major obstacle. An alternative to delivery of synthetic siRNA is in vivo expression from DNA templates. In the same paper, a functional short hairpin RNA (shRNA) targeted against luciferase was coexpressed in vivo from DNA templates by using an RNA polymerase III promoter. The expression of luciferase was inhibited by up to 98% in three independent experiments. These findings indicate that plasmid-encoded shRNAs can induce a potent and specific RNAi response in adult mice.

It is too early to speculate about the potential use of RNAi in therapy. However, in vivo RNAi may be applied in functional genomics or in identifying targets. This could be a more promising system than gene-knockout mice because groups of genes can be simultaneously rendered ineffective without the need for time-consuming crosses. Moreover, in the future, use of inducible systems could allow the shRNA approach to be utilized for targeting lethal genes or specific stages in development.

Interestingly, a recent publication shows that transgenic expression of shRNA in embryonic stem cells is functional and that the embryos derived from these cells[45, 46, 48] recapitulate a genetic null phenotype.

Summary and Outlook

Among the technologies available for knockdown in cell culture RNase H-dependent antisense oligonucleotides and RNAi are very popular, and for good reasons: both offer specificity and efficient knockdown; both are useful tools to study gene functions. Antisense and RNAi methods share many practical problems such as site selection, toxicity at high concentrations, and the difficulty of transfecting certain cell types. On the whole, overcoming the problems seems easier with siRNA than with As-ODN. The main reasons appear to be the lower concentration needed when using siRNA and the option to express siRNA within the cell. Therefore, in sensitive cellular models, RNAi seems to be more suitable for knockdown experiments and interferes less with subsequent functional readouts.

However, the antisense approach is a mature technology and countless research groups have years of experience of using it. In addition to RNase H-dependent knockdown, As-ODNs offer a

means to manipulate specific steps in mRNA processing, for example, splicing. RNAi, even though widely used for knockdown, is still in its infancy; we may encounter new problems or find new applications for this technique.

With the current knowledge one would probably choose RNAi to knock down a gene in a cell type sensitive to toxic agents. For those that do not want to advance the technology but simply wish to apply it, use of antisense oligonucleotides might be the method of choice to knock down genes in vivo. For the time being, it may be best to continue to watch both fields and choose the appropriate method on a case-by-case basis.

Acknowledgements

We would like to thank Maureen Spohn, Beatrice Schumann, and Anja Kissel for their excellent technical assistance and Lothar Hornung for oligonucleotide synthesis.

Keywords: antisense agents · gene expression · interference · knockdown · RNA

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Received: July 2, 2003 [A 708]

