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MECHANISTIC CHARACTERIZATION OF POST-TRANSCRIPTIONAL GENE SILENCING

Cecilia Dahlgren

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To my family

ABSTRACT

With the ongoing task of identifying and characterizing genes in the human genome, there is a great demand for tools to study these genes. Today, reverse genetics is the most effective way to explore gene function. This involves suppression of the gene of interest by a set of manipulation techniques. In addition to this, many conditions like viral infections, cancers and cardio-vascular diseases are caused by relative over-expression of certain genes in various cell types. Therefore, there is a great need for development of methods to suppress these unwanted gene expressions.

The work presented in this thesis concerns mechanistic studies of two alternative approaches to perform post-transcriptional gene silencing in mammalian cells: DNA-mediated antisense and siRNA-mediated RNA interference (RNAi). Today, both of these applications are used for gene regulation in either cell culture experiments or *in vivo* studies. Although some parts of the pathways of these two methods have been established, a great deal is still to be revealed about target specificity, knockdown efficiency and avoiding the anti-viral pathways generally occurring in mammalian systems.

In paper I, we determined the role of the rat GERp95 homologue to human Ago2. By targeting this protein with siRNA-mediated RNAi, the knockdown efficiency of the endogenous target gene NPY was substantially impaired. This determined the necessity of GERp95 for fully functioning RNAi. In paper II, a stable 3' cleavage product was discovered when targeting the mouse Fas gene by DNA-mediated antisense. This finding puts further light into the complexity of the RNase H-dependent antisense pathway and strengthens the need for careful selection of detection method when analyzing the outcome of post-transcriptional gene silencing in mammalian cells.

By a comparative analysis of siRNAs with varying chemical characteristics, we examined the mammalian anti-viral response system in paper III. From our results, we could conclude that LNA-modified siRNAs do not trigger IFN-dependent immune response in human HeLa cells. This in combination with the superior serum-stability and other potential advantages of LNA-modified siRNAs makes them a good candidate for future therapeutic applications. In paper IV we address the issue of siRNA specificity by performing a large-scale study of siRNA target variations. Finally we present some preliminary results regarding a putative novel method for visualization of miRNA action. Collectively, these studies have aimed to increase the general knowledge of post-transcriptional gene silencing in mammalian cells and they have more specifically investigated regulatory pathways, mRNA targeting and specificity for DNA-mediated antisense and RNAi.

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I Håkan Thonberg, Camilla Schéele, **Cecilia Dahlgren** and Claes Wahlestedt
Characterization of RNA interference in rat PC12 cells: requirement of GERp95
Biochemical and Biophysical Research Communications (2004) 318:927–934

II Håkan Thonberg, **Cecilia Dahlgren** and Claes Wahlestedt
Antisense-induced Fas mRNA degradation produces site-specific stable 3'-mRNA fragment by endonuclease cleavage at the complementary sequence
Oligonucleotides (2004) 14:221–226

III **Cecilia Dahgren**, Claes Wahlestedt and Håkan Thonberg
No induction of anti-viral responses in human cell lines HeLa and MCF-7 when transfecting with siRNA or siLNA
Biochemical and Biophysical Research Communications (2006) 341:1211–1217

IV **Cecilia Dahlgren**, Hong-Yan Zhang, Maria Grahn, Claes Wahlestedt and Zicai Liang
Analysis of siRNA specificity on targets with double-nucleotide mismatches
Submitted manuscript

CONTENTS

LIST OF ABBREVIATIONS

2'OM-ORN	2'O-methyl oligoribonucleotide
Ago	Argonaute
AS-ODN	Antisense oligodeoxynucleotide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
ISG	Interferon-stimulated gene
LNA	Locked nucleic acid
miRNA	Micro RNA
mi-RNP	miRNA-associated RNP
nt	Nucleotide
OAS2	2'-5'-oligoadenylate synthetase 2
ORN	Oligoribonucleotide
PS-ODN	Phosphorothioate antisense oligodeoxynucleotide
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RNP	Ribonucleoprotein
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
stRNA	Small temporal RNA
TLR	Toll-like receptor
UTR	Untranslated region

GENERAL INTRODUCTION TO THE THESIS

The backbone for all research within the field of molecular biology lies within the "The central dogma" which describes the basic processes occurring in living cells (Figure 1). Genomic DNA is transcribed into messenger RNA (mRNA) in the cell nucleus. The mRNA, which is then transported into the cytoplasm, codes for the subsequent translation of proteins. By the publication of the first draft of the human genome sequence in 2001(Nature, 2001), a large amount of newly identified genes with unknown properties increased the need for functional research tools. Today,

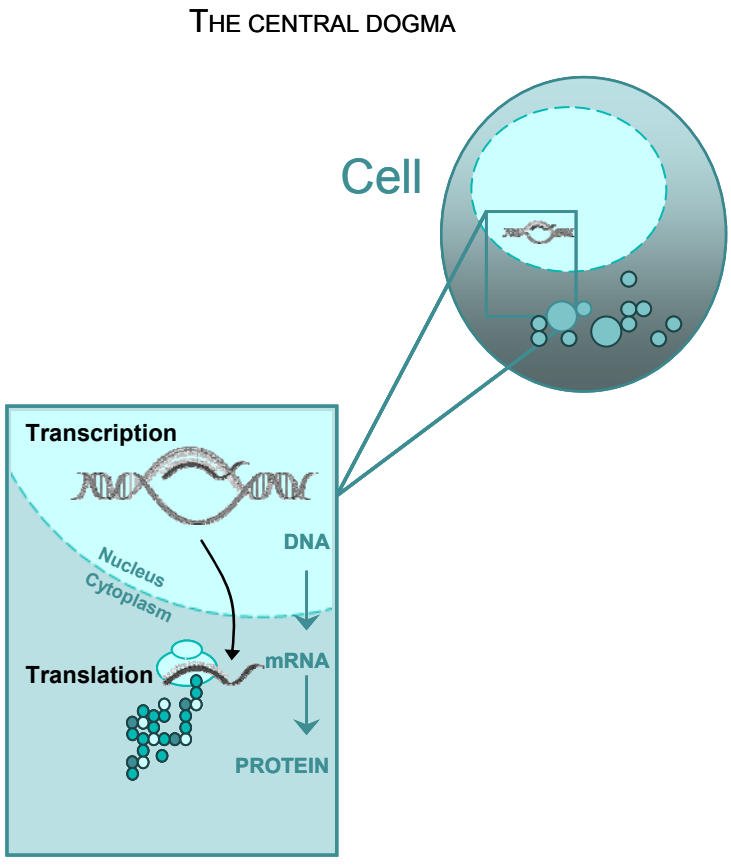

Figure 1. The backbone of molecular biology:
1. Replication of genomic DNA
2. DNA is copied to mRNA through **transcription**
3. Processed mRNA is transported from nucleus to cytoplasm
4. Protein is synthesized by the action of **translation**

reverse genetics is the most effective way to explore gene function. This involves suppression of the gene of interest by a set of gene manipulation techniques. In addition to this, many conditions like viral infections, cancers and cardio-vascular diseases are caused by over-expression of certain genes in various cell types. Because of this, there is a demand for effective methods to suppress this unwanted gene expression. There are three alternative pathways to influence gene expression – either on the transcriptional, post-transcriptional or post-translational levels. The work in this thesis has focused on the post-transcriptional level. Two methods for performing post-transcriptional gene silencing are DNA-mediated antisense and RNA interference (RNAi). These applications are both used for gene regulation either in cell culture experiments or *in vivo* studies. Although some parts of the pathways of these two methods have been established, a great deal is still to be further investigated.

The regulatory effects of DNA-mediated antisense was discovered in 1978 (Zamecnik & Stephenson, 1978). By using short single-stranded oligodeoxynucleotides (ODNs) complementary to the mRNA of a specific target gene, researchers were able to suppress the expression of the same gene. The technique was widely used for functional genomics studies and some successes were achieved for *in vivo* studies as well. Besides the relative sensitivity of the antisense molecules towards degradation within cells, additional difficulties regarding reproducibility of results and prediction of suppressible target regions were encountered. The foundation for the past decade's extensive utilization of RNAi-mediated gene silencing was laid when Andrew Fire and Craig Mello discovered that double-stranded RNA (dsRNA) could silence sequences complementary to its own when delivered to the nematode *Caenorhabditis elegans* (Fire et al., 1998). This revolutionary discovery awarded them the Nobel Prize in Physiology or Medicine in 2006. The response was found to be evolutionary conserved by subsequent observations in plants, protozoa, insects, mammals and fungi (Ngô et al., 1998; Cogoni & Macino, 1999; Hamilton & Baulcombe, 1999; Caplen et al., 2000; Hammond et al., 2000; Caplen et al., 2001; Elbashir et al., 2001b; Martin & Caplen,

2006). Also, small interfering RNAs (siRNAs) could be designed and used to silence genes in mammalian cells by mimicking components of the native RNAi pathway (Elbashir et al., 2001a).

Although the selection of efficient gene silencing siRNAs has been a lot easier than for the corresponding AS-ODNs, similar obstacles have been encountered when using them for *in vivo* studies. Additionally, secondary off-target effects caused by either specific motifs of the siRNA or the specific delivery method used have raised doubts about the specificity (Jackson et al., 2003; Jackson et al., 2006). The work presented in this thesis concerns functional aspects of DNA-mediated antisense and RNAi. We have tried to increase the general knowledge of post-transcriptional gene silencing and more specifically investigated regulatory pathways of mRNA targeting, specificity and cellular visualization of RNAi action. All cell culture-based work in this thesis has been performed in mammalian cell lines and the focus of this introduction will therefore mainly deal with post-transcriptional gene silencing in mammalian systems unless otherwise stated.

BACKGROUND

DNA-MEDIATED ANTISENSE

The finding that short antisense oligodeoxynucleotide molecules (AS-ODNs) could enter living cells and efficiently block expression of targeted genes created high expectations for this mechanism to become a valuable tool in molecular biology as well as in therapeutics as a functional suppressor of viral- and oncogenes (Zamecnik & Stephenson, 1978). Antisense oligos normally act by one of two mechanisms: 1 – annealing to its target RNA molecule and inhibit mRNA translation, either by restraining initiation of translation by steric hindrance or by preventing the splicing of pre-mRNA to become mRNA; or 2 – inducing mRNA cleavage through attraction of the endonuclease Ribonuclease H (RNase H) towards the hybridized DNA:RNA complex (Minshull & Hunt, 1986). This results in two fragments of mRNA, one lacking 5´-cap structure and one lacking 3´-poly(A) tail. The subsequent biochemical events are not fully understood but it is thought that the RNase H-mediated cleavage destabilizes the mRNA target, resulting in fragment degradation by exonuclease and endonuclease activities (Figure 2).

Nucleotide analogues

Following research provided further evidence that oligonucleotide delivery and subsequent target degradation or translational block in intact cells was a novel way to study gene function (Holt et al., 1988; Wickstrom et al., 1988). The data obtained however, showed large inconsistencies regarding efficiency of different oligonucleotides and the predicting good target suppression was difficult to achieve. These results made it obvious that several issues related to oligo degradation, cellular uptake and target recognition needed to be overcome before antisense molecules could be used as a robust and reliable tool for gene regulation (Wickstrom, 1986). This started an intensive search for chemical compounds that could enhance the efficiency and repress the negative traits of antisense molecules. At first, this turned out to be a difficult task – various modifications did indeed enhance both cellular

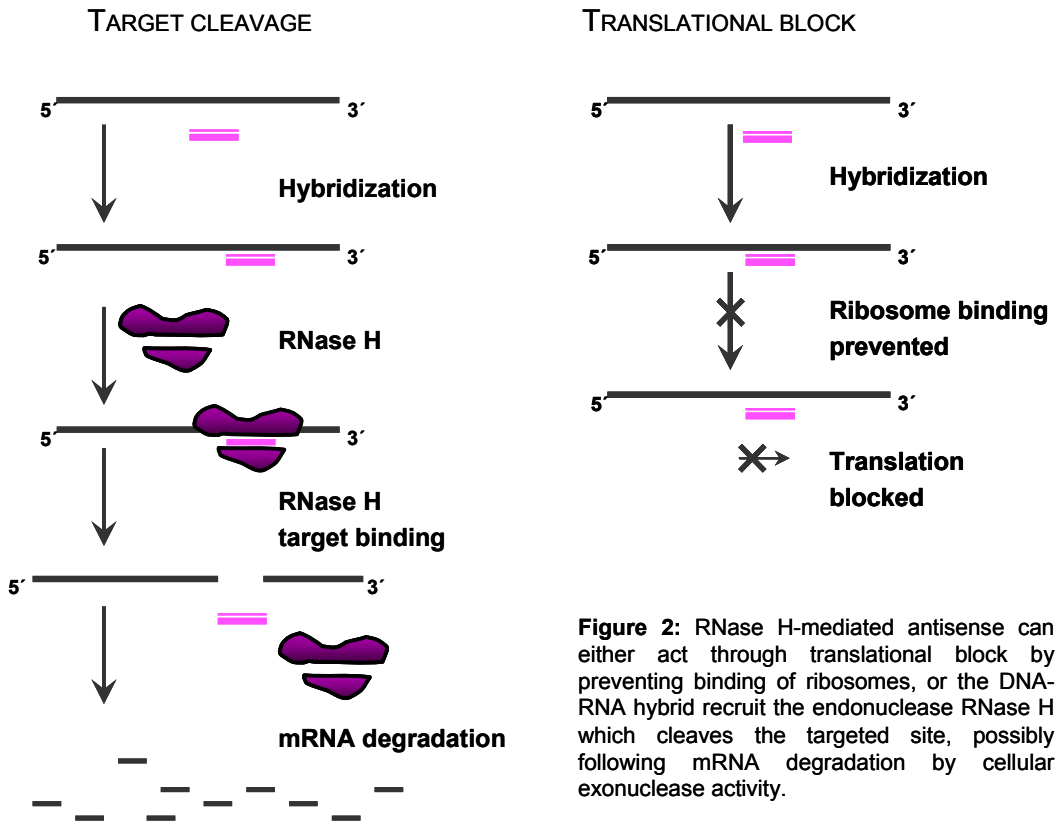

Figure 2: RNase H-mediated antisense can either act through translational block by preventing binding of ribosomes, or the DNA-RNA hybrid recruit the endonuclease RNase H which cleaves the targeted site, possibly following mRNA degradation by cellular exonuclease activity.

uptake and resistance towards nuclease activation – the drawback was however the loss of target degradation due to inability of the hybrid to form substrates for RNase H. To compensate for that, the oligonucleotide concentration needed to be raised in order to instead cause translational hindrance, with increased cytotoxicity as a consequence. Later on, terminal phosphoramidate modifications of single-stranded AS-ODNs proved to be an effective method for making them resistant to nucleolytic degradation caused by cellular exonucleases and that the DNA:RNA duplexes that where formed acted as substrates for RNase H (Dagle et al., 1990). Phosphorothioate oligodeoxynucleotides (PS-ODNs), where one of the nonbridging oxygens in the backbone have been replaced with sulfur, have also been widely used because of its enhanced serum stability. Phosphorothioate modifications of antisense oligos are able to recruit RNase H action but are not fully active in terms of suppression efficiency. Several PS-ODNs have reached clinical trials and the first antisense molecule approved as a therapeutic drug against cytomegalovirus retinitis was Vitravene (ISIS2922) (Geary et al., 2002).

Accessibility

One of the greatest challenges in antisense history has been the task to predict suppressible target sites. Several methods have been created to predict antisense accessibility – that is to what extent the AS-ODN can access homologous sequences of the target mRNA (Matveeva et al., 1997; Ho et al., 1998; Allawi et al., 2001). The mRNA accessible site tagging (MAST) technology is a wet lab approach where immobilized mRNAs are tagged with a randomized oligonucleotide library. This technique proved to be an effective tool for predicting effector molecules for a given target mRNA (Zhang et al., 2003).

Although many improvements of AS-ODN were made in the field of DNA-mediated gene silencing, the difficulties of creating molecules with satisfactory knockdown efficiency – together with issues like toxicity, delivery and most importantly the inability to get the procedure to work satisfyingly *in vivo* – still prevented it from becoming the useful tool as was first anticipated (Levin, 1999). These facts in combination with the discovery of RNAi in the late 90's, lead to a rapid decline of its use in molecular biology. Today, most of the work with antisense technology has shifted towards RNAi-mediated gene silencing conducted by siRNAs or short hairpin RNAs (shRNAs).

RNA INTERFERENCE

The process of RNAi is a naturally occurring regulatory pathway in all eukaryotes, where endogenously expressed long double-stranded RNA (dsRNA) molecules are processed by the endoribonucleases Drosha and Dicer (Bartel, 2004). The resulting 21-23 nt long RNA effector mediates post-transcriptional gene silencing by recruitment of a ribonucleoprotein complex (RNP) and sequence-specific attraction to its corresponding mRNA target. Depending on the distinct features of different RNA effector molecules, the inhibition of gene expression is either mediated through mRNA cleavage or translational repression. The finding that exogenously delivered dsRNA could trigger RNAi lead to a revolution in the field of post-transcriptional

gene silencing and the ability to study gene function. Initially, the use of RNAi was thought not applicable in mammalian systems since long dsRNA induces sequence-unspecific gene silencing by activation of anti-viral defence pathways (Kumar & Carmichael, 1998). Small interfering RNA molecules (siRNAs) were later discovered to avoid the degradational actions of these pathways (Elbashir et al., 2001a). The knowledge since then have been subjected to extensive research and found to be more complex than initially thought (Jackson et al., 2003; Sledz et al., 2003; Kim et al., 2004). Nevertheless, these double-stranded RNAs have been recognized as an efficient tool for achieving knockdown of gene expression and for functional genomics studies. There is also a great hope for this technique as a future tool for therapeutic applications.

RNAi mechanism

RNAi can either act as a response to endogenously expressed or exogenously delivered dsRNA. Endogenously expressed dsRNAs in form of pri-micro RNAs (pri-miRNAs) are processed in the nucleus by Drosha to become pre-miRNAs. These pre-miRNAs are subsequently transported to the cytoplasm of the cell where the endonuclease Dicer further processes them into 21-23 nt long mature miRNAs with 2-nt 3'-end overhangs (Bernstein et al., 2001). Following recruitment of the RNP complex, only one strand – the guide/antisense strand of the RNA duplex – is incorporated and leads the complex to its target mRNA sequence. The RNP consists of several members of the Argonaute (Ago) protein family. Exogenously delivered long dsRNA are processed in a similar manner as miRNAs in *C. elegans* and the fruit fly *D. melanogaster* in terms of Dicer cleavage. In mammals the exogenous RNA molecules usually consist of short hairpin RNAs (shRNAs) or small interfering RNAs (siRNAs), which are processed in a similar manner but the RNP complex taking care of siRNA-mediated gene silencing is termed RNA-induced silencing complex (RISC) (Hammond et al., 2000). To some extent, the RNAi mechanism is carried out differently for these molecules. RNAi caused by siRNAs usually acts by sequence-specific cleavage and subsequent degradation of the mRNA target molecule. The slicing activity of the target molecule is carried out by the Argonaute

2 (Ago2) component of RISC (Elbashir et al., 2001a; Liu et al., 2004; Meister et al., 2004). The actual cleavage site is located 10 nt downstream of the 5'-end of the siRNAs guide strand. On the other hand, miRNA-driven RNAi rather acts by occupying specific regions of the target mRNA, thereby preventing initiation of translation (Figure 3).

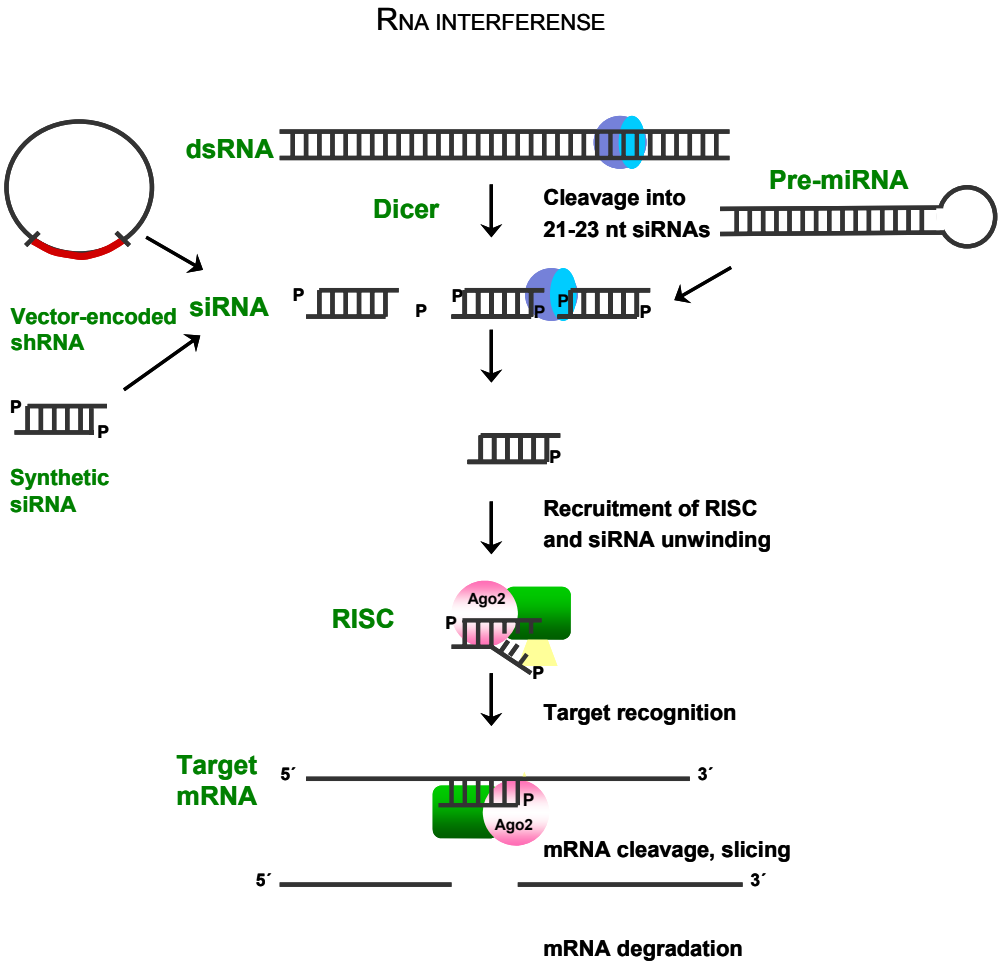

Figure 3. RNAi mediated by exogenously delivered siRNAs or shRNAs and endogenously expressed miRNAs in mammalian cells. Following processing by Dicer – depending on delivered molecule – the RNA duplex is unwound and the antisense strand incorporated into RISC. Subsequent target recognition leads to cleavage of target site and mRNA degradation or translational repression.

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. Dicer contains two RNase III motifs, a RNA helicase domain and a dsRNA-binding domain, and the enzyme performs an ATP-driven cleavage of long dsRNA to 21-23 nt long RNA effector molecules (ref). Ago-2, one of the RISC

components, has been identified as the catalytic engine responsible for the subsequent cleavage of target sequence, which ultimately leads to degradation of unprotected mRNA (Liu et al., 2004; Meister et al., 2004; Okamura et al., 2004).

Exogenously delivered RNA effectors

The mechanisms of RNAi were first discovered in *C. elegans* in the late 90's (Fire et al., 1998). The usage of synthetically produced siRNAs was later discovered to function in mammalian systems (Caplen et al., 2001; Elbashir et al., 2001a). Two variants of exogenously delivered RNA effector molecules are synthetic siRNAs or vector derived shRNAs. If comparing them to miRNAs, siRNAs have total sequence complementarity between both strands whereas miRNAs usually are interrupted by internal mismatches. Synthetic siRNAs can act on any given target mRNA molecule but miRNAs tend to act on the 3' untranslated regions (UTRs) of their targeted molecules. Furthermore, siRNAs usually generate target degradation whereas miRNA more commonly cause translational repression. This is however not always the case and siRNAs have been shown to act in a miRNA like way and vice versa (Doench et al., 2003; Saxena & Jónsson, 2003; Zeng et al., 2003). Discrepancies in actions between RNA effectors might be the result from various characteristics of the flanking sequences of their respective target transcripts. Similarly recruited enzyme complex might act equally towards their targets but differ in the subsequent actions. In terms of origin, miRNAs derive from transcripts of genomic regions other than protein-coding genes, whereas siRNAs usually are obtained from transposable elements, viruses or synthetic dsRNA.

The main advantage of using siRNAs in favor of shRNAs is the ability to control the transfected amount of molecules and thereby its subsequent uptake into RISC. These characteristics might have a large impact on the putative off-target and anti-viral effects that partly depend on the siRNA concentration. In contrast, the largest drawback of siRNA usage is the relatively short duration of achieved gene silencing. This feature is crucial when trying to affect the expression of proteins with a long turnover period – the siRNA-mediated silencing of the target gene might already

have ceased to act before any change in protein levels are detectable. When using RNAi for phenotypic studies and observing RNAi efficiency on a translational level, this might result in false negative results.

The detection of endogenously expressed miRNAs inspired the researchers to imitate the actions of miRNA-dependent RNAi by introducing synthetic genes expressing transcripts similar to miRNA precursor molecules. These molecules called shRNAs, are basically siRNAs linked together with a loop sequence and containing additional promoter and termination sequences. To become a functional RNA effector, this molecule has to be processed within the cell. The shRNA can also be expressed within cells by inserting them into viral vectors or plasmids. The shRNA-containing vectors are transcribed from RNA polymerase II or III promoters and the resulting transcript can be of pri-miRNA or pre-miRNA character for subsequent processing by Drosha/Dicer. The great advantages with this application are the abilities for long-term mediation of RNAi and co-expression of marker trans-genes to facilitate identification of shRNA-expressing cells. On the negative side we have the difficulties of controlling the amount of expressed RNA effector molecules from these vectors, possibly leading to saturation of the cells natural RNAi machinery and/or generation of non-specific responses. The possibility of integration of DNA sequences coding for these shRNAs, into the genome of the targeted cell, also poses a non-desirable cause of action (Huppi et al., 2005).

The preferred method for functional studies of a certain genes has to be assessed individually for each occasion. The applications of siRNA and shRNA-mediated gene silencing are to be considered as being complementary to each other. Several aspects like choice of cell line and preferred RNAi duration has to be taken under consideration. It is also important to optimize the experimental conditions regarding choice of transfection method, transfection time and amount of accessible siRNA to maximize knockdown efficiency and minimize off-target effects and non-specific actions. Pools of siRNAs or shRNAs targeting several positions of the same transcript might provide a more robust knockdown outcome and evaluating all of

these effector molecules by their individual capacity to suppress its target gene gives a clearer picture when studying phenotypic variations(Kawasaki et al., 2003; Myers et al., 2003).

Endogenously expressed RNA effectors

The action of miRNA-driven RNAi is the intrinsic cellular system to regulate gene expression. Many miRNAs only act in certain stages of cell development and there are also differences in expression depending on tissue or cell types (Bartel, 2004). Examples of miRNA functions are involvement in fat metabolism, cell proliferation, differentiation and apoptosis (Reinhart et al., 1999; Brennecke et al., 2003). MiRNAs have also been shown and suggested to be involved in several human diseases (Denli et al., 2004; Gregory et al., 2004; Pfeffer et al., 2004; Sullivan et al., 2005). Endogenous miRNAs act as silencing effectors in absence of direct sequence complementarity to its target. This feature makes these molecules able to affect the regulation of multiple genetic targets which share certain sequence similarities.

Primary hairpin structured miRNA transcripts (pri-miRNAs) are processed by the nuclear protein Drosha. The resulting ~60-70 nt long pre-miRNA is transported into the cytoplasm by an Exportin-5-dependent pathway (Lee et al., 2003; Yi et al., 2003; Zeng et al., 2003), where it is cut to a mature miRNA by Dicer (Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001). The discovery that the *C elegans* gene *lin-4* coded for a pair of small RNAs instead of an actual protein, paved the way for the later finding that miRNAs, originally called small temporal RNAs (stRNAs), are part of the RNAi pathway (Reinhart et al., 2000; Lee et al., 2003). This was proven by siRNA-mediated knockdown of the dsRNA-cleaving enzyme Dicer. Suppressed expressions of this enzyme lead to the accumulation of a precursor molecule to an already established miRNA – *let-7* (Hutvágner et al., 2001). Similarly, this was shown for the *C elegans* homologue Dcr-1 to mammalian Dicer (Grishok et al., 2001; Ketting et al., 2001; Knight & Bass, 2001).

RNAi-induced gene silencing performed by miRNAs is usually a consequence of partial homology of the miRNA towards the 3' untranslated region (UTR) of its target molecule. It has also been shown that miRNA precursor molecules can provide as ancestor for several miRNAs (Lee-Huang et al., 1993) and a single RNA target can be regulated by several different miRNAs (Doench & Sharp, 2004). The resulting decrease in protein levels is caused by translational repression rather than target degradation. This is verified by the absence of change on mRNA levels and these miRNAs prevent protein translation by either hindering the initiation of translation or the protein elongation (Olsen & Ambros, 1999; Seggerson et al., 2001; Humphreys et al., 2005; Pillai et al., 2005). The level of sequence complementarity for each miRNA is however varying and some miRNAs are capable of mediating cleavage in a siRNA-like manner (Hutvágner & Zamore, 2002).

A number of computational approaches have been created to estimate the number of miRNA genes and miRNA targets in the human genome. These predictions state that approximately 1 – 5 % of the total protein-coding genes are miRNA genes. Furthermore, 30 % of all protein-coding genes are thought to be regulated by miRNAs (Lewis et al., 2005). Until today 533 miRNA sequences have been validated for *homo sapiens (Ambros et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006).*

Silencing potency

Delivery

An important factor for efficient gene silencing by RNAi is the type of delivery. A popular method for efficient siRNA delivery is the use of cationic lipids, which consists of a positively charged head and a hydrophobic tail. When mixed with nucleic acids, the cationic heads of the lipid heads interact with the phosphate backbone, which result in the formation of lipoplexes (lipid-DNA/RNA). These complexes are then easily transported by endocytosis through the negatively charged cell surface (Elouahabi & Ruysschaert, 2005). The use of lipofilic agents has however been difficult to use for *in vivo* studies. The relatively short time span of lipoplex stability also poses an obstacle for using this for putative therapeutic

applications. To overcome this problem, method that use lyophilized lipid-siRNA complexes have been developed with sustained knockdown efficiency as a result and on the same time prolonged stability with possible usefulness as a high-throughput screening tool and for therapeutic approaches (Andersen et al., 2007). Other methods include shRNA-expressing lentiviral constructs – which are infected into cells through certain packaging vectors, or electroporation (Ventura et al., 2004). Both the transfectability of different cell lines and the specific delivery method have an impact on the efficiency of siRNA delivery. It is of great importance to optimize the transfection conditions for each cell line and each type of RNAi effector molecule. To establish the transfection efficiency, one can use fluorescently labeled molecules for microscopal visualization, but the most reliable tools for analyzing this are studies on mRNA and protein level of RNAi function (Huppi et al., 2004, 2005). Choice of method for analyzing knockdown efficiency is also an important aspect to reflect upon. Measurement of mRNA level by e.g. real-time PCR does not necessarily need to correlate to the actual amount of translation for a specific protein in a given cell.

Design

A key feature is the ability for each RNA effector to perform knockdown of its specific target. Much of the knowledge about the specificity of synthetic siRNAs comes from observations of mi-RNP assembly and target recognition. The importance of the thermal stability of the 5' region of a RNA effector molecule was discovered to be of great importance for strand selection and subsequent loading into RNP (Khvorova et al., 2003; Schwarz et al., 2003) When RISC is assembled, the choice of strand incorporated into the complex is nearly always the one strand that has least thermal stability of the 5' end. It is thought that a helicase residing in the RISC takes care of the unwinding of the siRNA strands and selectively chooses the end which is most easily unwound (Khvorova et al., 2003) (Schwarz et al., 2003; Silva et al., 2003). Some reports have however made evident the occurrence of equally stable miRNA strands, resulting in similar uptake into mi-RNP and possible functionality of them both (Lagos-Quintana et al., 2002; Krichevsky et al., 2003; Schwarz et al., 2003). When designing siRNA for efficient knockdown of a specific

target, a crucial step is to make sure the 5' end of the antisense sequence is more loosely paired than the 3' end.

Another key feature for siRNA specificity is the 5' end sequence complementarity to its mRNA target. Positions 2-8 of metazoan miRNAs, the so called "seed" region, have been proven important e.g. by evidence of species conservation, perfect complementarity to 3' RNAi mediating UTR regions (Lai, 2002; Brennecke et al., 2003; Lewis et al., 2003; Stark et al., 2003; Lewis et al., 2005), and experimental evidence of enhanced specificity of this region for siRNA-mediated RNAi (Jackson et al., 2003; Pusch et al., 2003), Paper IV). The explanation for this phenomenon is not certain but one suggestion is that this region is responsible for RISC target-recognition and that remaining sequence complementarity decides whether following actions consist of translational repression or target degradation (Bartel, 2004). Furthermore, the potential target cleavage following recognition takes place between nucleotides 10 and 11 counting from the 5' region of the complementary siRNA (Elbashir et al., 2001a; Elbashir et al., 2001b). As a consequence of this, perfect or near-perfect base-pairing of a within this region of a siRNA towards its mRNA target is essential for subsequent gene silencing, as verified by experimental studies (Du et al., 2004).

Besides the various factors described above, the silencing efficiency of an RNA effector molecule is also largely dependent on the relation between available amount of silencing complexes and their respective target RNA. This adds another dimension to RNAi effectiveness by including possible cellular interactions separate from them involved in the actual pathway.

Specificity

It is generally not recommended to use large amounts of siRNA even though good knockdown efficiency can be achieved by this method. Pushing the limits of the targeted cells and its RNAi machinery are likely to cause non-specific events of various kinds (Doench & Sharp, 2004; Haley & Zamore, 2004).

Anti-viral responses

The innate immune system posed a difficult task for researchers in early stages of RNA-mediated gene silencing in mammalian systems. Long dsRNA causes the activation of interferon (IFN)-regulated pathways as being part of mammalian cells' innate defense against viral attack. This process involves sequence-independent triggering of the type I interferon-response via up-regulation of IFN-α and IFN-β (Samuel, 2001). Subsequent actions include activation and up-regulation of numerous signaling cascades and interferon-stimulated genes (ISGs) respectively. The 2'-5'-oligoadenylate synthetase 2 (OAS2) is such a gene that upon activation produces 2'–5'-oligoadenylate molecules. These molecules bind to and thereby trigger RNase L dimerization (Zhou et al., 1993), which is believed to mediate cellular apoptosis by genome-wide RNA degradation and inhibition of anti-apoptotic processes (Chawla-Sarkar et al., 2003). As a parallel response dsRNA-dependent protein kinase (PKR), which is also thought to be related to the up-regulation of IFN-β, is autophosphorylated, which in turn stimulate the phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α). This in turn leads to numerous actions each contributing to global inhibition of translation (Srivastava et al., 1998).

The discovery that certain RNAi pathways were conserved also in mammals and that long dsRNA could mediate target-specific knockdown in mammalian embryonic cell lines, encouraged the researchers that the anti-viral actions in a cells response to dsRNA might actually be overcome. The key finding was that dsRNAs molecules shorter than 30 nucleotides were effectively knocking down gene expression in a sequence-specific manner without activation of PKR (Caplen et al., 2001). In a very short time period, 21-nt long siRNAs became a widely used tool for target-specific knockdown of gene expression (Elbashir et al., 2001a; Yang et al., 2001). Because of the relatively high costs of chemically synthesized siRNAs molecules, enzymatically synthesized siRNAs became very popular. The 5' triphosphate group generated by this method was however shown to induce substantial increase in IFN-α and IFN-β levels hence leading to the conclusion that siRNAs constructed in this manner was not appropriate as a reliable tool for studies of functional genomics (Sledz et al., 2003; Kim et al., 2004).

INFLAMMATORY PATHWAYS INDUCED BY LIPOFILIC DELIVERY

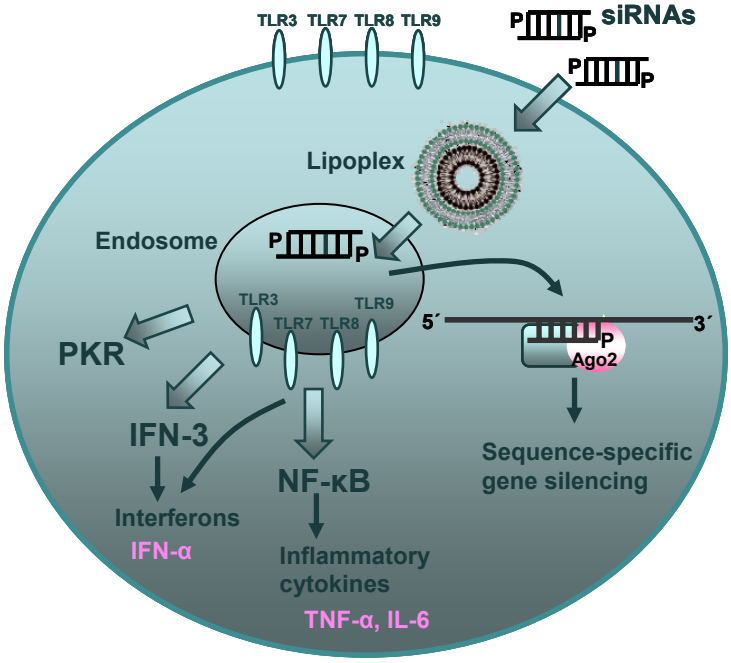

Figure 4. When transfecting siRNA with cationic lipids, they are compartmentalized into endosomes which contain immunostimulatory receptors that possibly can be triggered by sequence-specific motifs in certain siRNAs (modified from Sioud 2005).

More recent studies of human cells have proved that induction of inflammatory cytokines is also triggered by chemically synthesized siRNAs and that the response is sequence-dependent and requires endosomal compartmentalization achieved when transfecting cells with lipofilic agent (Figure 4) (Hornung et al., 2005; Judge et al., 2005; Sioud, 2005). Cytokines are triggered by the activation of Toll-like receptors (TLRs), which are commonly present in endosomes (Medzhitov et al., 1997; Aderem & Ulevitch, 2000; Armant & Fenton, 2002). SiRNAs containing these immune-stimulatory motifs does not induce any inflammatory actions when delivered through electroporation (Sioud, 2005). To avoid this immune recognition of siRNAs, there are at least three different suggested strategies available (Sioud et al., 2007): 1 – to choose a delivery agent that do not involve endosomal compartmentalization (Song et al., 2005), 2 – to use chemical modifications of specific nucleotides that block immune activation but simultaneously stabilizes the siRNA with retained silencing

efficiency (Fedorov et al., 2006; Sioud, 2006), 3 – to inhibit endosomal maturation by anti-inflammatory agents (Sioud et al., 2007).

Off-target effects

Although siRNAs with full sequence-complementarity towards its target is considered to be substantially more efficient than mismatched ones, microarray studies of mRNA regulation have shown that far from full complementarity is needed (Jackson et al., 2003). The 5'-region of the siRNA strand is least tolerable towards sequence variations (Haley & Zamore, 2004). Detailed reporter assays of siRNA-targeting has furthermore proved the importance of sequences 5-11 as being an extra sensitive region in terms of siRNA-target mismatches (Du et al., 2004), Paper IV. The importance of the nucleotide positions 2-8 "seed" region for miRNA target recognition also influence siRNA design. To avoid any possibility of siRNA off-target effects, careful analysis should be made to avoid miRNA-like base pairing to 3' UTR regions as a consequence of sequence similarities to miRNA seed regions (Doench et al., 2003).

As discussed above in the siRNA potency section, the thermal stability of the 5' end of the antisense strand is of great importance for knockdown efficiency. This feature also influence siRNA specificity since efficient loading of the antisense strand into RISC minimizes the occurrence of RISC loading the sense strand. Even a small fraction of RISC-sense strand complexes could give cause to unwanted off-target effects. A novel method has been developed to further optimize antisense strand loading into RISC, where the siRNA molecule consists of one intact antisense strand and two complementary sense strand separated by a nick. This nick mimics the natural occurring event of Ago 2 cleavage after RISC loading and is supposed to help following destruction of the sense strand. Indeed, these small internally segmented interfering RNAs (sisiRNAs) show increased potency and specificity towards their target mRNA (Bramsen et al., 2007). In addition to the recommendations above, minimizing the amount of effector molecules could substantially reduce the probability for off-target effects in any given cell system (Fedorov et al., 2006).

Nucleotide analogues

Even though siRNA-mediated RNAi has become an effective method for studying gene expression in many cell types, the progress for *in vivo* applications has been hampered by numerous difficulties regarding delivery, nucleotide stability and targeting specificity. To overcome this, efforts have been made to increase the siRNA's resistance against degradation by insertions of backbone alterations or modifications of the 2'-OH group of the RNA sugar in either of the two siRNA strands. One example of chemical backbone modification is the phosphorothioate – which gives increased serum stability and increased silencing efficiency depending on insertional position (Parrish et al., 2000; Harborth et al., 2003). The locked nucleic acid (LNA) is another type of nucleotide analogue where a methylene bridge connects the 2′-oxygen with the 4′-carbon of the ribose ring. The bridge locks the ribose ring in a RNA-like 3′-*endo* conformation (Wahlestedt et al., 2000). LNA-modified siRNAs (siLNAs) gain increased specificity and nuclease resistance without activation of the anti-viral response system in comparison to regular siRNAs (Elmén et al., 2005), paper III). 2' uridine modifications of siRNAs make these molecules evade the innate immune system and furthermore enhance their specificity in terms of off-target effects (Sioud, 2006). The 2'-O-Methyl modification of the RNA pentose sugar is a well tolerated alteration if limited to a few positions of the siRNA strand (Holen et al., 2003). These few modifications can increase the knockdown efficiency and durability of a siRNA. Modifications of this type make the RNAi effectors more resistant towards nuclease activity and thus increase the stability (Czauderna et al., 2003). Moreover, insertion of nucleotide analogues into specific sites can increase the probability of preferred strand uptake into RISC, thereby reducing the risk for unspecific effects.

In vivo and therapeutics

Although proven an effective tool for *in vitro* knockdown of almost any targeted gene, RNAi has yet to prove its functionality for *in vivo* and therapeutic applications. Promising studies have been made but the efforts now lie in the ability to combine the target gene specificity-enhancing modifications with functions that increase

siRNA delivery and direction towards certain cells or organs. As for *in vitro* studies, the choice is between using mature siRNA molecules or by introducing DNA-based shRNA-expressing vectors. Because of the potential risk of integrating foreign DNA into the targeted genome and the difficulty of controlling the effector molecule amount for the latter method, most *in vivo* experiments are performed with synthetic siRNAs.

Delivery

To achieve efficient administration of siRNAs for *in vivo* studies, methods like local injection and systemic delivery have been used. When using local injections a smaller amount of RNAi effector molecules are usually needed than for systemic administration. On the downside, this invasive method is limited to the organs or tissues available for this type of action. Systemic administration usually involves the use of high-pressure tail vein-injections of substantially larger volumes (Lewis et al., 2002). Besides being relatively expensive and the inability to perform this method in humans, high-pressure administration is considered to cause side effects (Zhang et al., 2004). Both naked siRNA and formulations involving aptamer-conjugates, liposomes, polymers or antibodies have been used in various studies with effective results (Reich et al., 2003; Soutschek et al., 2004).

Overall process

To create an efficient and specific siRNA for subsequent *in vivo* applications, a few guide lines have been set; 1 – the siRNA composition has to be determined by using one of the computational siRNA-prediction tools available on the internet. These predictions minimize the possibilities of causing non-specific events like triggering the immune system or suppression of non-targeted genes; 2 – chemical modifications of the antisense and/or sense strand need to be introduced for increased siRNA potency, specificity and nuclease resistance; 3 – the siRNA need to be validated *in vitro* for the specific traits listed above; 4 – an appropriate method for delivery need to be selected depending on targeted tissue etc. To minimize unwanted side-effects like activation of the immune system and non-specific down regulation of non-targeted genes, chemical modifications are recommended to be introduced into the

siRNA duplex. As an example, modifications of the 2' sugar of certain nucleotide positions effectively increase siRNA specificity and phosphorothioate alterations of the 3' terminal ends of both strands greatly increases the siRNA stability. In addition to this, when designing siRNAs to use for *in vivo* and putative therapeutic applications, it is preferred to select targets that are evolutionary conserved throughout species to facilitate transitions from *in vitro* to *in vivo* and later clinical studies (De Fougerolles & Vornlocher, 2007).

RNAi in clinical studies

The first *in vivo* evidence for RNAi-mediated treatment were reported in 2003 (Song et al., 2003)and the first clinical studies on RNAi-based therapeutics came a few years later. Following the fast-moving development of RNAi methodology, a number of RNAi-based therapeutics is currently under clinical studies. Two of them aim to treat age-related macular degeneration (AMD) causing blindness and another one targets the respiratory syncytial virus (RS-virus) (Bitko et al., 2004).

AS-ODN VS. siRNA

What specific consequences is then the reason for the decline of RNase H-dependent antisense agents for the benefit of the explosive increase of siRNA-mediated RNAi? If we compare the mechanistic pathways they differ completely for the active molecules – siRNA is recruited by RISC following antisense strand uptake and target cleavage by Ago 2. AS-ODNs on the other hand acts by annealing to the target mRNA and thereby cause RNase H-mediated cleavage. Also, RNase H-mediated cleavage is considered to take place in the cell nucleus on pre-mRNA level whereas siRNA-mediated RNAi is carried out in the cytoplasm on mature mRNA level (Figure 5) (Martinez et al., 2002; Zeng & Cullen, 2002).

The most striking difference between antisense and RNAi is the double-stranded feature of siRNA and the single-stranded feature of AS-ODN. Some comparison of siRNAs and various sets of modified AS-ODNs have stated that similar silencing

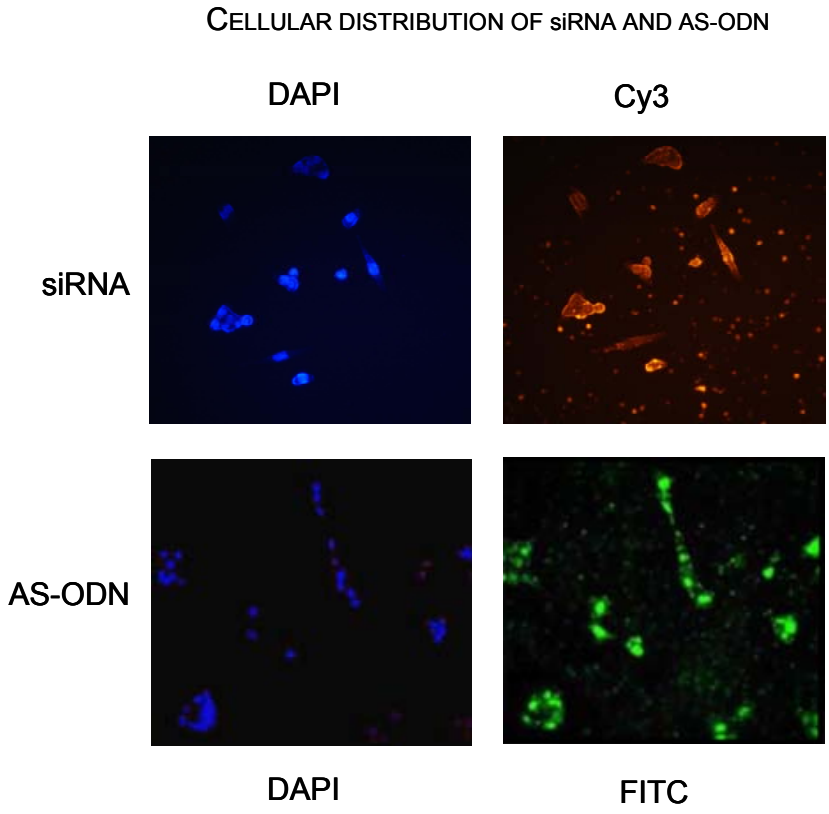

Figure 5. Microscopal view of siRNA and AS-ODN distribution after cellular transfection. Upper images show DAPI nuclear staining to the left and Cy3-labeled siRNA to the right. Bottom images show DAPI nuclear staining to the left and FITC-labeled AS-ODN to the right. These images clearly visualize the tendency for siRNA to accumulate in the cytoplasm and the opposite action of AS-ODNs to accumulate in the nucleus.

efficiencies can be achieved under optimized conditions. Target position for both of these applications has proven to be of great importance. Many of the functional sites for siRNAs are equally good for antisense molecules and vice versa, which implies that mRNA secondary structure is clearly important for target accessibility of both siRNAs and AS-ODNs. This is however not always the case and these discrepancies could possibly be explained by certain preferences for the different nucleases active in RNase H-mediated antisense and siRNA-mediated RNAi (Vickers et al., 2002). Furthermore, pre-mRNA (containing introns) and mRNA appears to be the targets for RNase H and siRNA respectively and structural differences between these two might be the reason for the observed differences in activity. It has also been suggested that RNase H-dependent activity appears to have a slightly earlier onset than that of RNAi (Vickers et al., 2002). The fact that RNase H acts on pre-mRNA might be useful in some cases when targeting of all transcriptional variants is important. Reversely, selected targeting towards specific transcripts could be crucial for other applications and thereby favor the use of RNAi. In regards of toxicity, AS-

ODNs have been reported to cause more severe toxic effects at significantly lower levels of delivered molecules, than siRNAs of corresponding sequence (Leaman, 2005).

Similarities

In addition to the differences between AS-ODNs and siRNAs there are also some striking similarities. Both of the molecules can be chemically modified to increase beneficial properties and these modifications are necessary for efficient *in vivo* applications (Soutschek et al., 2004; Morrissey et al., 2005). They can cause destruction of target RNA, and when distributed systemically *in vivo* they localize to the liver (Braasch et al., 2004). Additional similarities of features like duration of action and knockdown efficiency have been observed, but for AS-ODNs this is only achieved with backbone modifications like 2'-MOE which gives a significant raise in nuclease resistance for these molecules (Monia et al., 1993).

The observation that siRNA-mediated RNAi has taken over most of the research efforts in mediation of post-transcriptional gene silencing in mammalian cell systems speaks for its superior use of action properties. The fact that it mimics the cells own system to regulate gene expression to fight off viral infections implies that it might be favorable in the long run. Despite the issues described in this work – like un-specific targeting of non-targeted genes (paper IV), stimulation of immune regulatory pathways (paper III) and problems with intracellular stability – great progress have been made to overcome these obstacles and today there lies great promise in the efforts of using siRNA-mediated gene silencing as a future therapeutic drug. On the other hand, a possible explanation for the fast evolution of siRNA approaches in comparison with antisense oligos is that previous work with antisense-mediated gene silencing paved the way for a much faster development of siRNA as a tool for both *in vitro*, *in vivo* and clinical studies. Many of the obstacles encountered in the siRNA world, had already been overcome in the antisense era. The great advantage for siRNA so far has been the increased stability that the double-stranded siRNA stand for in comparison to the single-stranded antisense oligo. *In vivo* however, similar difficulties have been encountered as for the usage of AS-ODN and no improved

actions of potency has been discovered. In fact, antisense oligos have some distinct features that theoretically make them more favorable for use in therapeutics than siRNAs: 1 – they have half the molecular weight; 2 – they do not require hybridization after being synthesized, facilitating large-scale preparations; 3 – they have no known action in native cellular regulatory processes, making them less prone to induce toxicity as for some of the RISC-competing siRNAs (Grimm & Kay, 2006).

AIMS

The aims of my thesis work has been to explore and put further light into the field of post-transcriptional gene silencing by investigating pathways and mechanisms of single-stranded DNA-mediated antisense and miRNA/siRNA-induced RNAi in mammalian cell lines. The specific aims for each paper were:

I To investigate the role of the rat Argonaute protein GERp95 for RNAi in rat PC12 cells.

II To determine the down-regulating properties of phosphorothioate-modified AS-ODNs in terms of mRNA target degradation in mouse AML12 cells.

III To characterize the anti-viral mechanisms in response to transfections with enzymatically or chemically synthesized siRNAs with or without LNA-modifications in human cell lines HeLa and MCF-7.

IV To develop and optimize a high-throughput method for generation of a siRNA target-library and to use this technique for investigating siRNA specificity towards targets with double-nucleotide mismatches.

In addition to this, we wanted to create a system for exploring miRNA functions like cellular distribution and site of actions when mediating RNAi (preliminary results).

RESULTS AND DISCUSSION

PAPER I

Investigation of GERp95 function in rat PC12 cells

At the time, a number of functions and the composition of RISC had been determined – including the involvement of several proteins from the Argonaute protein family. Previous research had investigated the function of Ago2 in *D melanogaster* and the human equivalent eukaryotic initiation factor 2C2 (eIF2C2) (ref). In order to determine the role of the rat homologue Golgi-ER protein 95 kDa (GERp95) to human eIF2C2 and its involvement in RNAi, we designed siRNAs targeting the mRNA of GERp95 and the endogenously expressed neuropeptide Y (NPY) in rat PC12 cells. These effector molecules were validated for functionality of suppression activity. Sequential knockdown of first GERp95 and then NPY would determine the functional role, if any, for rat GERp95 in siRNA-mediated gene silencing.

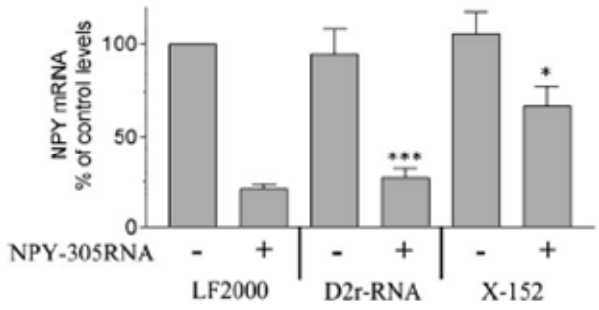

Figure 6. Pretreatment with siRNA targeting GERp95 inhibits further RNAi. PC12 cells grown in cultures were transfected with siRNA targeting GERp95 for 24 h followed by a transfection with siRNA targeting NPY for an additional 24 h. Shown is quantitative RT-PCR analysis of NPY mRNA after 24+24 h of transfection with 100nM siRNA.

All knockdown experiments were performed in rat PC12 cells, using lipofilic agents for transfection of chemically synthesized siRNAs. When selecting the siRNA target sequence for rat NPY, we used a prediction method available for antisense accessibility and combined these data with general siRNA design rules (Elbashir et al., 2001b; Zhang et al., 2003). The efficiency of target knockdown was measured on mRNA level by either quantitative real-time PCR for NPY and GERp95 or Northern blot for NPY. Protein expression levels were determined using Western blot and a rabbit anti-rat polyclonal antibody targeting GERp95.

From this study we established a robust system to achieve RNAi in cultured rat PC12 cells. By targeting NPY mRNA by a minimum amount of synthetic siRNA we managed to efficiently suppress gene activity measured on both mRNA and protein levels. From the real-time PCR and Northern blot data we could draw the conclusion that our siRNA was highly potent and able to guide mRNA target cleavage following subsequent degradation. The transfection efficiency was examined by ocular examination of Cy3-labeld siRNAs and maximum levels were observed already after 4 hours. As expected, the Cy3-labeled siRNAs were localized to the cytoplasm and the mRNA knockdown was shown to last for at least 96 hours. Additionally, by treating cells with the translation-inhibiting agent cycloheximide and observing the knockdown efficiency of NPY, we determined that RNA translation is not necessary for functional RNAi. To investigate the role of GERp95 – an Argonaute family protein – in rat PC12 cells, we targeted GERp95 mRNA with a siRNA. Sequential knockdown of NPY by siRNA was shown to be substantially impaired (Figure 6). By this we could conclude that the GERp95 protein is required for optimal RNAi functionality in rat PC12 cells.

PAPER II

mRNA target degradation by DNA-mediated gene silencing

Target-specific mRNA degradation using a classical DNA-mediated antisense approach results from hybridization of a short DNA-oligo to the target mRNA. The

formed DNA-RNA hybrid is recognized by endonuclease RNase H, which in turn cleaves the RNA strand into two fragments with a 5'-cap and a 3'-poly(A) tail respectively. These fragments are considered relatively unstable and were thought to be rapidly degraded by cellular exonuclease activity (Jacobson 1996). Far from all designed AS-ODNs are however functional target suppressors and in a validation experiment for the antisense accessibility method MAST, we discovered that DNA-mediated antisense can result in the accumulation of stable 3' cleavage fragments (Zhang et al., 2003).

Six phosphorothioate-modified oligonucleotides of various predicted functionality were examined for their ability to induce target-specific mRNA degradation. The cell surface receptor Fas was targeted in mouse AML12 cells by transfection with AS-lipoplexes. Fas mRNA levels were measured by Northern blot and ribonuclease protection assay (RPA).

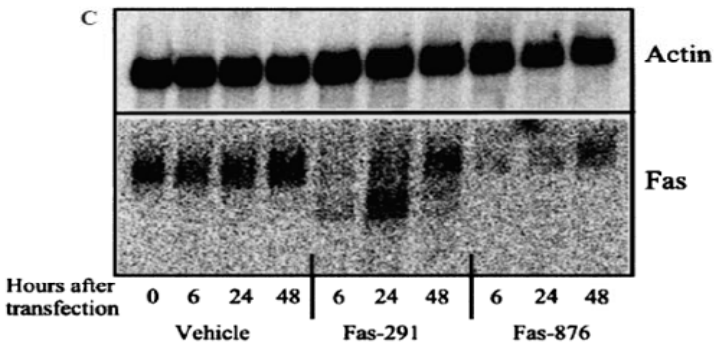

Figure 7. Northern blot of AML12 cells treated with AS-ODN for various times. Cells were treated with AS-ODN (200 nM) for either 6, 24, or 48 hours. Then, total RNA was isolated and subjected to Northern blot. Top, membrane hybridized with α-actin cDNA probe; bottom, membrane hybridized with Fas cDNA probe. Vehicle indicates cells treated with LF2000 alone.

As anticipated, four of the tested antisense oligonucleotides reduced the mRNA levels significantly. However, Northern blot analysis revealed that cells treated with one of the antisense molecules resulted in the accumulation of a band of lower molecular weight. Interestingly, this band was not detected after 6 hours of

transfection but instead appeared at 24 hours (Figure 7). To investigate this further, we designed a RNA-probe covering the targeted Fas mRNA (including the antisense target site). A fully protected RNA would result in a band size of 270 bp. Any cleavage fragments caused by incomplete target degradation would result in a shorter band. Following RPA analysis, using this specific AS-ODN revealed that total target degradation was achieved after 6 hours in accordance to Northern blot results. After 24 hours, a 20 nt shorter band appeared which determined it as being a stable fragment of the target mRNA 3'-end and that it originated from within the AS-ODN complementary sequence. The reason for this phenomenon is somewhat difficult to determine but might involve a sequence-/position-dependent saturation of exonuclease activity over time.

The implications of these results stresses the importance of carful selection and validation of DNA effector molecules since measured knockdown efficiency can vary greatly depending on which time points that are considered. Furthermore, this type of 3'-end mRNA fragments might serve as templates for the translation of truncated proteins affecting the function of the native full-length protein, possibly resulting in secondary off-targets effects. Additionally, when using quantitative real-time PCR, researchers must make sure to choose amplicon sites which span the predicted RNase H cleavage site to prevent false results.

PAPER III

siRNA-mediated activation of anti-viral responses

Initially, siRNA-mediated gene silencing was thought to be a highly specific event, avoiding the activation of cellular anti-viral responses. Some alarming reports, demonstrating the occurrence of certain unwanted off-target effects, raised the important issue of careful evaluation of siRNA specificity (Sledz et al., 2003; Jackson et al., 2006). This study represents a detailed analysis of putative anti-viral gene induction as a response to a range of siRNA in cultured cell lines. We wanted to examine the usefulness of LNA nucleotide analogues in siRNA molecules and its

possible influences of immune system activation. To do this we examined the up-regulation of IFN-β and OAS2 and the phosphorylation of eIF2α. IFN-β is part of the type I IFN-response pathway causing sequence-independent mRNA degradation. OAS2 is an ISG, which by activation triggers RNase L-mediated global RNA degradation. The phosphorylation of eIF2α is an immune responsive action, mediated by previous autophosphorylation of PKR (Figure 8).

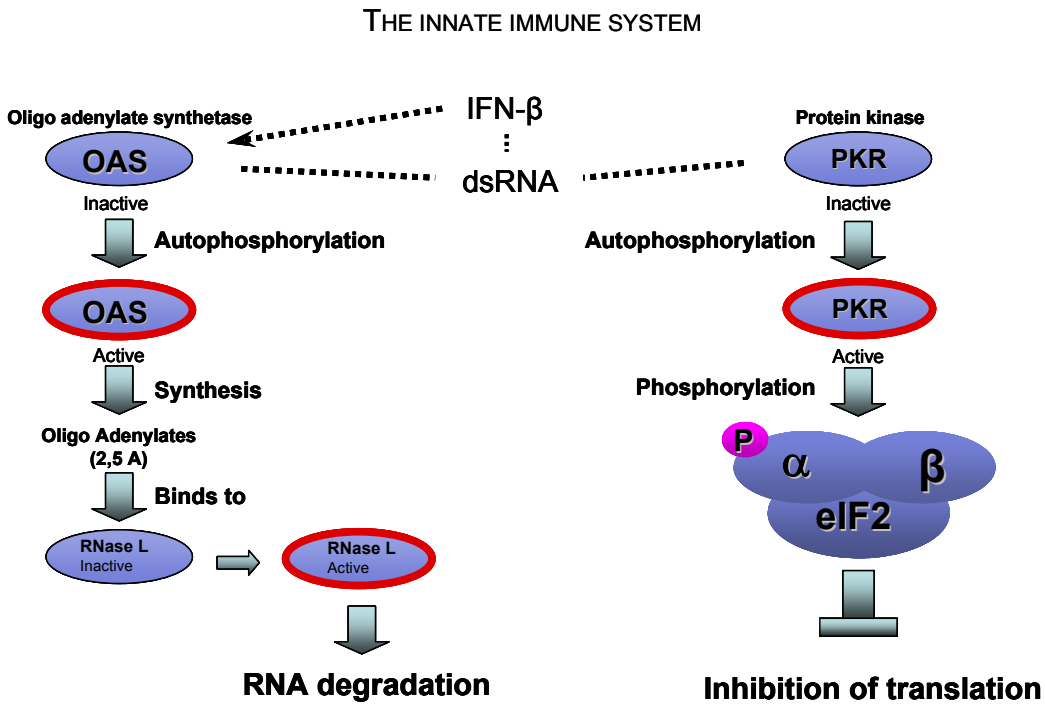

Figure 8. Activation of the innate immune system by long dsRNA or triphosphate-containing enzymatically derived siRNAs.

Chemically or enzymatically synthesized siRNAs were transfected into cultured human HeLa or MCF-7 cell lines. The outcome was analyzed and validated using quantitative real-time PCR on mRNA level and Western blot or ELISA on protein level. Quantitative real-time PCR is a relatively fast and sensitive technique, which only requires a small amount of RNA as starting material.

The results show that neither synthetic siRNA nor siLNA induce anti-viral response in HeLa and MCF-7 cell lines. This work also illustrates that whether a synthetic siRNA has an actual endogenous target in the host cell or not does not have an impact on its ability to induce anti-viral responses. By demonstrating clear responses to both long dsRNA and *in vitro*-transcribed siRNA we were able to contrast the differences seen from using synthetic siRNA molecules. Additionally, *in vitro*-transcribed siRNAs, which gave a dramatic increase in cellular IFN-β levels, had no effect on phosphorylation of eIF2α (Figure 9). This observation indicates that PKR-mediated phosphorylation occurs independently of IFN-β stimulation. We conclude that for the particular cell lines used in our experiments, both siRNA and siLNA are safe to use as a tool for reverse genetics in the aspect of IFN-dependent anti-viral gene response.

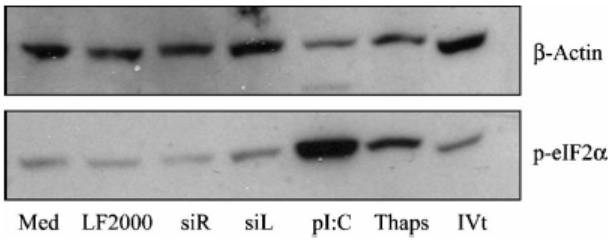

Figure 9. Western blot illustrating phosphorylation of eIF2α in cells transfected with pI:C and Thapsigargin. No increase in phosphorylation could be detected after transfection with *in vitro*-transcribed siRNA. HeLa cells were either transfected with Lipofectamine2000 (LF2000) alone, synthetic siRNA (siR), synthetic siRNA containing LNA nucleotides (siL), poly(I:C) (pIC), Thapsigargin (Thaps) or with *in vitro*-transcribed siRNA (IVt).

PAPER IV

siRNA target specificity

Although siRNAs as a tool for gene knockdown is a great promise for future applications, the specificity of siRNA-mediated gene silencing needs to be thoroughly investigated. Most research regarding siRNA specificity has involved analysis of affected off-target genes instead of exploring the specificity of RNAi

itself. In a previous study, a systematic analysis of the silencing effects of a siRNA towards a target site with all possible single-nucleotide mismatches had been performed (Du et al., 2004). In this large-scale study, we wanted to investigate this further by analyzing double-nucleotide mismatched target sites. To do this we deployed and optimized a high-throughput method for generation of a siRNA target library. Furthermore, we wanted to compare siRNA knockdown efficiency depending on whether the mutations are positioned in the siRNA itself or in the corresponding target site

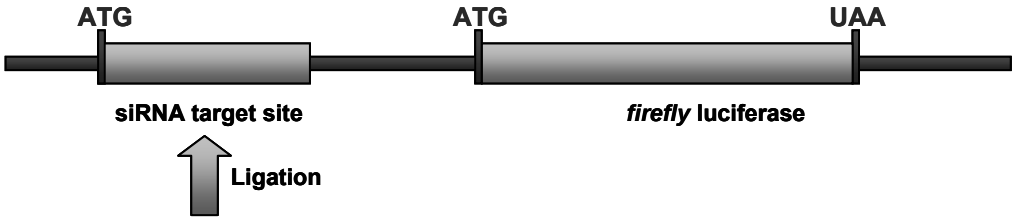

Figure 10. Schematic diagram of the fusion *firefly* luciferase reporter – siQuant. From the left is one in-frame ATG start codon, followed by a box representing the siRNA target site and the luciferase gene with another ATG codon to compensate for possible introduction of stop codons within the double-mutated target site.

A reporter-based siRNA validation system called siQuant was used to analyze different target sites by subjecting them to siRNA-mediated RNAi (Figure 10). In this study we combined the validation vector with a single-tube nucleotide Oligomix for generation of a double-mismatch siRNA-target library. The oligomix containing 1539 different variations of a 19 nt long siRNA target sites with double-nucleotide mismatch pairs, was PCR-amplified following restriction cleavage with appropriate enzymes. The short duplex DNA oligonucleotide corresponding to the target site of functional siRNA – siCD46 – was cloned in-frame upstream of the firefly luciferase gene in the linearized siQuant vector. Following transformation of competent cells, each positive colony was verified by Pyrosequencing. A total of 709 unique siQuant constructs were obtained by this method. These vectors were then used for co-transfection with siCD46 into HEK293 cells. The knockdown efficiency of the siRNA was then monitored by measuring *firefly* luciferase signaling by Dual-luciferase Reporter Assay System on a luminometer.

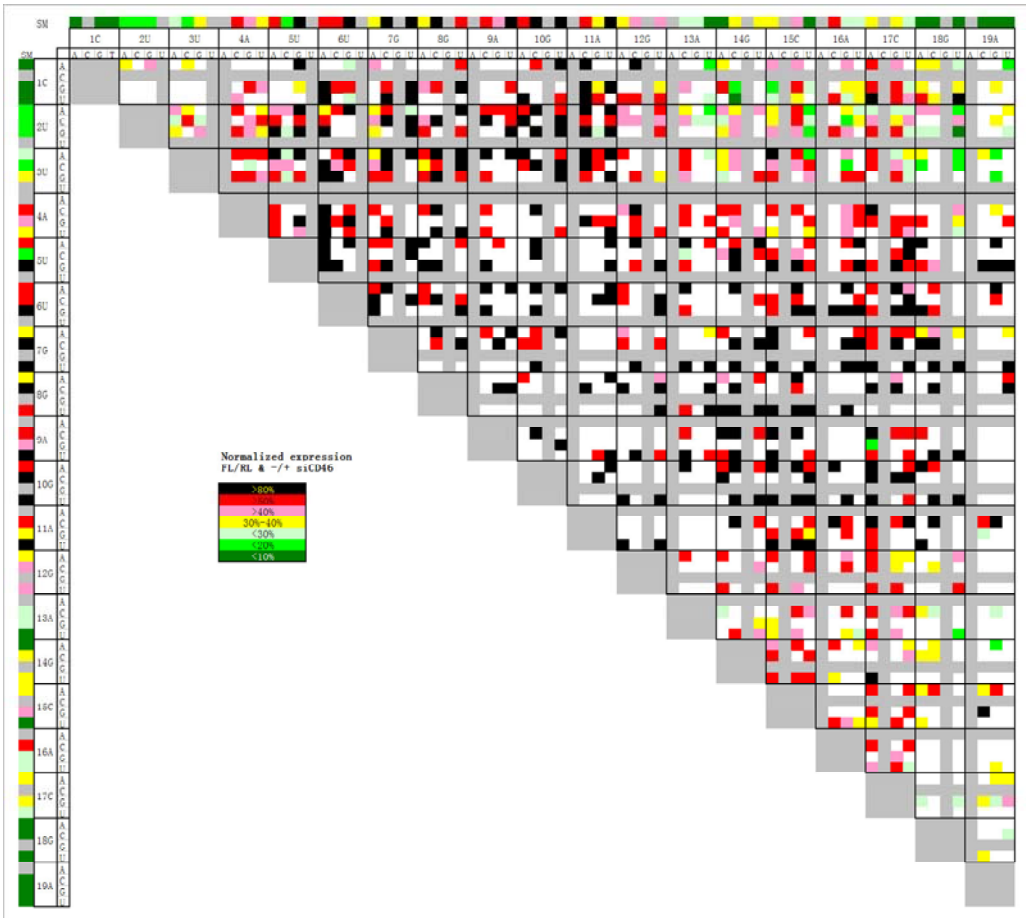

Figure 11. Colour-coded expression chart visualizing positional and regional trends for siCD46 knock down efficiency and tolerance towards double-mutations in its specific target site.

In this study we managed to utilize and optimize a straightforward an effective method for constructing a siRNA target library. By using this library we were able to perform a large-scale analysis of siRNA specificity which revealed that ~35 % of the double-mismatched siRNA target sites in our library were down-regulated by more than 50 % (Figure 11). This is a fairly large sum in terms of off-target activity and further strengthens the careful investigation of siRNA properties when selecting RNA effector molecules. Furthermore, we wanted to examine whether point-mutated siRNAs is a reliable tool for examining the specificity of a particular siRNA. Three unique siQuant constructs were selected and three siCD46 with corresponding mutations in the antisense strand were designed for this purpose. The results showed that nucleotide mismatches in the antisense strand of the siRNA molecule can give a different knockdown pattern when comparing them to a wild type siRNAs ability to

suppress expression from target sites with corresponding mutations. Target site selection has proven to be critical for RNAi approaches to get effective gene knockdown. The recommendations for siRNA selection that are available today are not fully validated and a lot can still be improved. The results from this study have given additional valuable information on RNAi specificity and can hopefully add parameters to aid in the construction of computational tools predicting siRNA efficiency.

PRELIMINARY RESULTS

Intracellular actions of miRNA-mediated RNAi

MiRNAs are small, 21- to 23-nucleotide non-coding RNAs, and the cell's own system to control gene expression by either mRNA degradation or translational repression. While some of the early steps of the RNAi pathways are more or less defined, the downstream mechanisms by which miRNAs act as a mediator of target degradation/translational repression are not yet fully known. In this project we wanted to perform a microscopal study to explore the actions of miRNA. To give further light into these events and to explore the cellular functions of miRNA action in a visual context, we constructed a RNA molecular beacon that could act as a target for a non-endogenous miRNA in mammalian HeLa cells. A molecular beacon is a 30-40 bp long oligonucleotide labeled with a fluorophore and a quencher. The oligo consist of a stem region which keeps the fluorophore and quencher together as long as there is no sequence available complementary to the linking loop region. In our case, a correctly hybridized oligo will form a hairpin structure with the miRNA target site forming a loop (Figure 12). When the miRNA of interest finds this target, the oligo will unwind and the quencher and fluorophore separated from each other, resulting in a measurable signal. With this technique we hoped to visualize miRNA-mediated RNAi, including cellular localization, clustering, compartmentalization and site of action for translational regulation.

Methods

A molecular RNA beacon containing a miRNA-target for *firefly* luciferase (miR-lucT beacon) was designed and validated for correct secondary structure with RNA*fold* (ref). The 5'-end starts with a 7 nt stem sequence followed by a 21 nt long target site for luciferase miRNA (miR-luc) and one extra nucleotide to create optimal secondary structure. The miRNA-target sequence follows by the complementary 7 nt stem sequence. The 5' end was labelled with the fluorescent molecule Cy5 and the 3'-end with its corresponding quencher BBQ650.

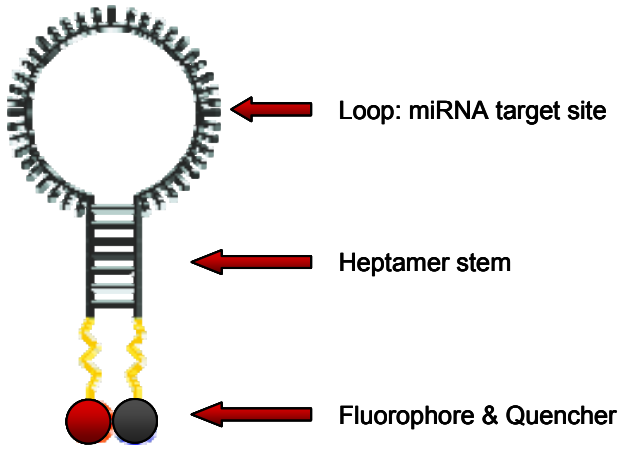

Figure 12. RNA molecular beacon consisting of 7 nt stem region, linked together with a miRNA target site and labeled with 5' Fluorophore and 3' Quencher.

Human HeLa cells cultured in chambered coverglass slides (Nunc) the previous day, were subjected to transfection of the RNA beacon together with GFP-reporter vectors coding for miR-luc and miR-neg (negative control). The cell density was ~50 % at the time of transfection and the transfection reagent Lipofectamine2000 (Invitrogen) was used at a concentration of 0,2 %. Media was changed 4 hours after transfection and the total transfection time was 24 hours. MiR-lucT beacon was transfected either alone or in combination with the miR-luc expressing vector, or the negative control vector. Following microscopal examinations were carried out at time points 0h, 2h,

4h and 24 hours post-transfection. Confocal images were obtained using a Leica TCS SP confocal laser-scanning microscope (Leica Microsystem).

Results and discussion

The transfection of double-stranded RNA into mammalian cell lines, poses an evident risk of nucleotide degradation caused by cellular regulatory/defense mechanisms. We hypothesized that the 7 nt double-stranded feature of our RNA beacon was too short to be recognized by Dicer.

The results show that the RNA beacons are efficiently delivered into HeLa cells using Lipofectamine2000 as transfection agent. However, the miR-luc expressing vector could only be detected in ~20 % of the cells and detectable GFP levels occurred after 24 hours. The RNA beacon started to fluoresce already after 2 hours (data not shown), even in the absence of the miRNA-expressing vector corresponding to our target beacon (Figure 13). No difference could be seen between cells containing both miR-lucT beacon and miR-luc vector and cells containing only miR-lucT beacon (Figure 14). The beacons seemed to cluster into large vesicles outside of the cell nucleus. Whether the fluorescent signal was a result from

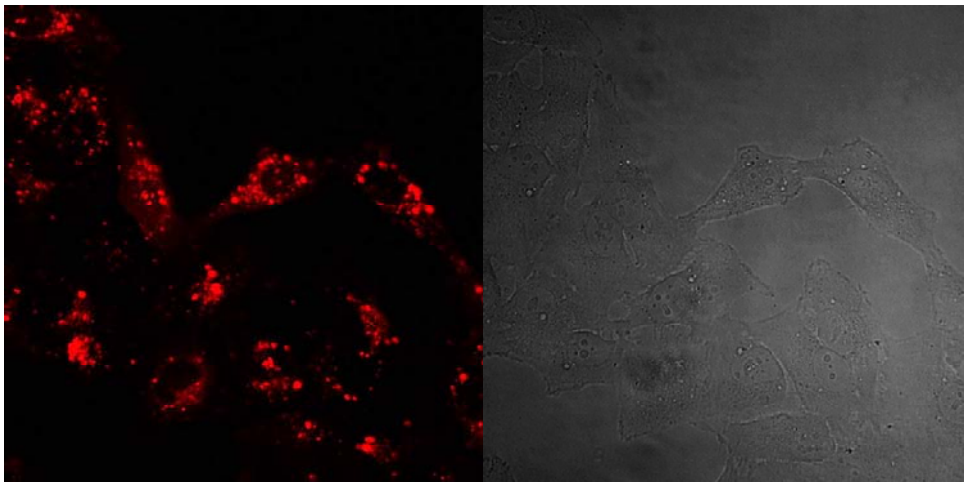

Figure 13. Hela cells transfected with miRT-luc beacon at time point 4 hours after transfection.Left pane showing Cy5 signalling.

degradation of the beacon (possibly caused by Dicer) or simply caused by denaturation of the beacon stem, making the quencher unable to inhibit Cy5 signal, can not be determined at this time.

The initial experiments with the miR-target beacons we constructed to visualize miRNA distribution during RNAi, thus failed to perform as anticipated. There are however other strategies we can adopt to enable these RNA beacons to function properly and to further examine the intracellular distribution of miRNA action. First, another type of transfection method can be examined. Endosomal compartmentalization of double-stranded RNA can trigger sequence-dependent immune responses as discussed previously. To rule out that this response is not the cause of the degradation/separation of strands for our RNA beacon, a transfection

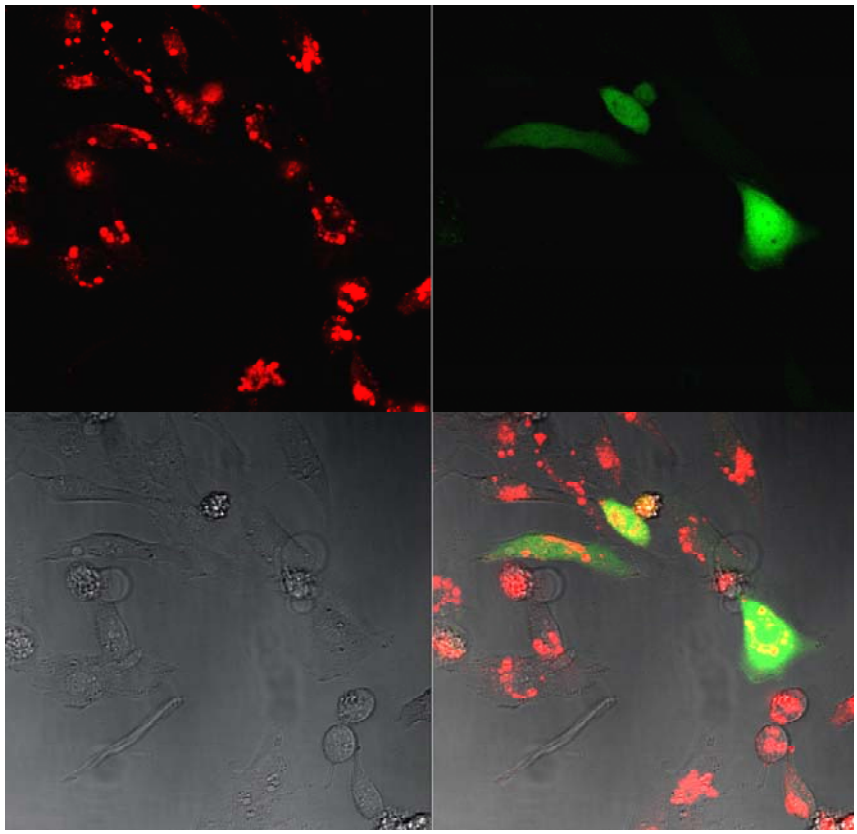

Figure 14. Hela cells transfected with RB-miR-t-luc and miR-luc at time point 24 hours after transfection. Red signal is Cy5 from RNA beacon and and green is GFP from miR-luc expressing Vectors.

method like electroporation could be validated. Second, there is a various set of nucleotide backbone modifications available to improve the stability and nuclease resistance of double-stranded RNA within living cells. Oligonucleotides entirely consisting of the 2'-O-methyl modified versions have been shown to inhibit RNAi function by acting as target for and irreversibly bind to the antisense-containing RISC. This occupation of the RNAi-mediating complexes prevents them from attaching to their actual targets (Hutvágner et al., 2004). One possibility would be to modify selected positions within the stem region of the RNA beacon with this type of nucleotide analogue. The advantage with this procedure would be to stabilize the RNA beacon towards nuclear degradation if that is reason for the observed Cy5-signalling in our experiments. The key issue would of course be to stabilize the molecule enough to not be degraded by cellular endonuclease and exonuclease activity but not so much that miRNAs targeting the RNA beacon-loop would be prevented from unwinding the stem.

CONCLUDING REMARKS

The discovery of RNAi as a naturally occurring mechanism for controlling gene expression provided a powerful tool for functional genomic studies and has dramatically increased our knowledge of gene regulation. In addition to this, the ability of mimicking this native process is promising for future therapeutic applications. The first enthusiastic years after the original breakthrough were followed by some alarming setbacks when it was revealed that the specificity of the effector molecules was not as good as first anticipated. Furthermore, the transition from cell culture-based systems to *in vivo*-applications progressed in a relatively slow pace. Researchers came across similar obstacles as encountered for the preceding usage of DNA-mediated antisense.

With our research we have tried to contribute to the general understanding of the underlying mechanisms of DNA-mediated antisense and RNA-mediated RNAi. The results from paper I provided a detailed analysis of the kinetic actions of siRNA-mediated gene silencing in rat PC12 cells. The selected siRNA caused complete degradation of its target mRNA by using very low concentration of effector molecules. Full knockdown effect was observed already after 12 hours and was shown to be sustained for at least 94 hours. Furthermore, we investigated the role of the rat GERp95 protein in PC12 cells. By targeting the expression of this protein by siRNA-mediated RNAi, we could see that the subsequent knockdown of the endogenously expressed NPY was greatly impaired. We thereby established the function of GERp95 as being part of the RNAi pathway in rat PC12 cells and the necessity of this protein for optimal gene silencing. In paper II we examined the targeting process of DNA-mediated antisense. We observed a time-dependent accumulation of a stable 3' fragment of the target mRNA resulting from antisense-mediated cleavage by the endonuclease RNase H. From these results we could conclude that RNase H-dependent mRNA targeting does not always result in complete degradation and that DNA-mediated antisense is a complex process with various regulatory pathways.

To examine the mechanistic actions of siRNAs with various characteristics and chemical modifications from a immuno-stimulatory point of view, we monitored the putative up-regulation of the ISGs IFN-β and OAS2 in paper III. Our research showed that the IFN-β responsive pathways in human HeLa and MCF-7 cell lines were not triggered by transfection with chemically synthesized siRNAs with LNA-modified nucleotides. The lack of phosphorylation of eIF2α when transfecting with enzymatically synthesized siRNAs that cause up-regulation of IFN-β, suggests that these immuno-responsive pathways act independently of each other. Later findings have since then revealed that there are other anti-viral responses in the cell that can be triggered by siRNAs. The TLRs is a set of receptors that activate a cytokine-regulated inflammatory response caused by endosomal compartmentalization of siRNAs. This response was shown to be an event depending on distinct features of siRNA sequence-composition.

To further investigate the siRNA specificity, we deployed a high-throughput method to facilitate large-scale analysis of siRNA target specificity. A surprisingly high amount of siRNA target sites with various combinations of double-nucleotide mismatches were substantially degraded by the corresponding siRNA. Additionally, we showed on differences in knockdown efficiency depending on whether the point-mutations were inserted into the siRNA target site or the siRNA itself. This data confirmed the usefulness of using the siQuant reporter construct as an effective tool for examination of siRNA target specificity. Finally, some preliminary results investigate a putative novel method for microscopical detection of miRNA and visualization of miRNA-driven actions within a cell. The initial results point at difficulties in maintaining intracellular stability for this RNA beacon but with proper modifications of the nucleotide backbone, it might become an elegant tool for miRNA detection.

Summarizing, the results presented in this thesis has contributed to the general understanding of post-transcriptional gene silencing in mammalian cells by investigating different aspects of DNA-mediated antisense and RNAi. More

specifically, by the identification of stable degradation products as a result of antisense recruitment of RNase H, we have verified the importance of using multiple detection methods when examining knockdown efficiency on mRNA level. We have also strengthened the advantages of using LNA-modified siRNAs in terms of avoiding the activation of IFN-β dependent anti-viral response. Finally, in terms of siRNA specificity, our data further stresses the need for careful sequence analysis of siRNA/miRNA to avoid off-target effects, before any attempts on using them as therapeutic agents can be made.

POPULÄRVETENSKAPLIGT PERSPEKTIV

En människa består av miljarder olika celler som alla innehåller en egen uppsättning av vår arvsmassa – det mänskliga genomet. Arvsmassan består av ca 20 000 – 25 000 proteinkodande gener som ligger till grunden för utvecklingen av vår biologiska karaktär. Generna överförs från generation till generation och består av sammanlänkade nukleinsyror i form av dubbelsträngat DNA. ”Det centrala dogmat” ligger till grund för alla processer av biologisk karaktär och innebär att DNA-molekyler som befinner sig i cellkärnan, kopieras till ett budbärar-RNA (mRNA) som är enkelsträngat. Det i sin tur transporteras ut till cellens cytoplasma och ansvarar där för nybildning av proteiner. Proteinerna är uppbyggda av aminosyror och de tar hand om alla vitala processer i levande organismer.

Sedan 2003, då kartläggningen av det mänskliga genomet blev slutfört, har man fått tillgång till ett enormt material med sekvensbestämd information av olika gener. Ett stort behov av att funktionsbestämma dessa gener har därmed uppkommit. För att vidare utforska hur de bidrar till en komplett organism med specialiserade vävnader och organ, används ett antal metoder för att påverka dess uttryck. Ett mycket vanligt förfaringssätt att påverka en gens uttryck är att tysta ner den. För att tysta ner en gen kan man använda dess budbärar-RNA som måltavla. Genom att avlägsna en gens budbärar-RNA hindras också dess motsvarande protein från att bildas. Resultatet kan studeras på olika nivåer och ger användbar information om en gens funktion genom att studera vad som händer när den inte uttrycks. Detta fenomen kallas för att påverka geners uttryck på en post-transkriptionell nivå.

Två av de metoder som används för denna funktion kallas antisens och RNA-interferens (RNAi). Molekylärbiologisk forskning utförs ofta med hälp av experiment i cellkulturer och för antisens-tekninken används en liten enkelsträngad DNA-molekyl som motsvarar en del av sekvensen för det mRNA som man vill göra sig av med. När denna DNA-molekyl förs in i cellerna binder den till mRNA-molekylen vilket i sin tur rekryterar ett antal proteiner som ser till att mRNA bryts

ner. Den andra tekniken – RNAi – upptäcktes i slutet av 90-talet av 2006 års Nobelpristagare Craig Mello och Andrew Fire. RNAi är från början en naturlig mekanism som använder sig av små dubbelsträngade RNA-molekyler som kallas mikro-RNA (miRNA). Dessa miRNA medverkar i ett antal processer som ger ett liknande resultat som antisens. Naturligt RNAi svarar för ett stort antal funktioner i olika celltyper. Förutom huvuduppgiften att reglera genuttrycket i olika cellstadier, kan denna mekanism motverka virusangrepp och skadliga effekter orsakade av sk ”hoppande gener” – transposoner. För molekylärbiologisk forskning kan man utnyttja detta fenomen genom att tillverka syntetiska små RNA-molekyler (siRNA) som imiterar miRNA att utföra RNAi. SiRNA-molekylen utformas så att det riktar sig mot just den gen du vill inaktivera. Många sjukdomstillstånd orskaras av ett överuttryck av vissa gener. Detta har lett till stora förhoppningar om att på medicinskt vis kunna använda siRNA för att stänga av sjukdomsalstrande gener i celler för bl a cancer, virusangrepp och hjärt- och kärlsjukdomar.

Idag testas flera potentiella läkemedel som är baserade på antisens och RNAi. Det första antisens-baserade läkemedlet blev godkänt för behandling 1998. Inga RNAi-baserade läkemedel har godkänts ännu men tekniken i sig är relativt nyupptäckt och detta innebär att det finns mycket kvar att lära. Förmodligen kommer det dröja ytterligare ett antal år innan tekniken får sin verkliga genomslagskraft som terapeutiskt alternativ. Vissa problem för båda ovanstående tekniker har påträffats vad gäller identifiering av verksamma RNA-/DNA-molekyler. Utöver detta har det visats att siRNA kan stimulera till en anti-inflammatorisk reaktion och därmed orsaka oönskade effekter hos värdcellen. Andra studier har visat på skillnader i specificitet för olika siRNA, vilket innebär att andra gener än dem man vill inaktivera också kan påverkas. Det övergripande syftet med studierna i denna avhandling har varit att öka förståelsen för och studera hur antisens och RNAi fungerar som mediatorer av post-transkriptionell genreglering. En ökad kunskap om deras respektive mekanismer kommer förhoppningsvis utöka dess användbarhet som forskningsredskap och underlätta för framtida användning som verksamma läkemedel.

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