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RNA Interference-Based Therapeutics: Harnessing the Powers of Nature

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1. Introduction

The central dogma of biology describes the transfer of biological information from DNA through to protein (Crick, 1970). In the first phase, known as transcription, DNA is converted into a complementary sequence of messenger RNA (mRNA). This mRNA allows the genetic message to be communicated outside of the nucleus, to other areas of the cell, where it is then translated into protein by ribosomes.

Most human diseases arise from increased function or dysfunction of proteins within the body. Since these proteins are generated from DNA via mRNA, modulation of this flow of genetic information can convey a therapeutic effect on the disease state. Mammalian cells possess the genetic instruction to make 50,000 to 100,000 different proteins but only 10-20% of these are found in any single cell. Therefore, a gene must contain instructions for the regulation of the production of protein in correct amounts and at the correct time for each cell type. Gene regulation is one of the most complex molecular processes known, involving up to 10% of the proteins that cells produce.

In 1998, Andrew Fire and Craig Mello described RNAi as an endogenous gene expression pathway activated by double-stranded RNA (dsRNA) in the worm *Caenorhabditis elegans*. For this pioneering work, Fire and Mello were awarded the 2006 Nobel Prize in Physiology or Medicine. The discovery of the natural RNAi mechanism for sequence-specific gene silencing launched a new era in antisense technology. During the 1990s, a number of gene-silencing phenomena that occurred at the posttranscriptional level were discovered in plants, fungi, animals and ciliates, introducing the concept of post-transcriptional gene silencing (PTGS) or RNA silencing (Baulcombe, 2000; Matzke et al., 2001).

The most important technologies for gene suppression are: antisense oligonucleotides, aptamers, ribozymes and RNA interference (RNAi). The first report that gene expression could be modulated by the use of reverse complementary (antisense) oligonucleotides was made in 1978. Antisense molecules are synthetic segments of DNA or RNA, designed to mirror specific mRNA sequences and block protein production, these molecules are designed to inhibit translation of a target gene to protein via interaction with mRNA. Aptamers are single-strand DNA or RNA oligomers, which can bind to a given ligand with high affinity and specificity due to their particular 3-D structures and thereby antagonize the biologic function of the ligand. Recent developments demonstrate that aptamers are valuable tools for diagnostics, purification processes, target validation, drug discovery and therapeutics. Ribozymes are

enzymes that are generally considered to be comprised of RNA, which can act as catalysts as well as genetic molecules. They cleave a target RNA, inhibiting the translation of RNA into protein, thus stopping the expression of a specific gene. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. RNAi is a naturally occurring regulatory mechanism present in most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules to direct homology-dependent gene silencing. Two types of small RNA molecules -microRNA (miRNA) and small interfering RNA (siRNA)- are central to RNA interference. RNAs are direct gene products, and these small RNAs can bind to other specific mRNA and either increase or decrease their activity, for example by preventing a messenger RNA from producing a protein.

2. Mechanism of RNA interference

The mechanism of RNAi is initiated when long dsRNA are processed to siRNAs by the action of an RNase III-like protein known as Dicer (Bernstein et al., 2001; Hammond et al., 2000) (Figure 1). The resultant siRNA are 21 to 24 nucleotides in length, double stranded and have 3′ overhangs of 2 nucleotides (Stevenson, 2004). Exogenous synthetic siRNA or endogenous expressed siRNAs are incorporated into the effector complex known as RNA-induced silencing complex (RISC), where the antisense or guide strand of the siRNA guides RISC to recognize and cleave target mRNA sequences (Elbashir et al., 2001) upon adenosine-triphosphate (ATP)-dependent unwinding of the double-stranded siRNA molecule through an RNA helicase activity (Nykanen et al., 2001). The catalytic activity of RISC, which leads to mRNA degradation, is mediated by the endonuclease Argonaute 2 (AGO2) (Liu et al., 2004; Song et al., 2004). AGO2 belongs to the highly conserved Argonaute family of proteins. Argonaute proteins are ~100 KDa highly basic proteins that contain two common domains, namely PIWI and PAZ domains (Cerutti et al., 2000). The PIWI domain is crucial for the interaction with Dicer and contains the nuclease activity responsible for the cleavage of mRNAs (Song, et al., 2004). AGO2 uses one strand of the siRNA duplex as a guide to find messenger RNAs containing complementary sequences and cleaves the phosphodiester backbone between bases 10 and 11 relative to the 5' end of the guide strand (Elbashir, et al., 2001). An important step during the activation of RISC is cleavage of the sense or passenger strand by AGO2, removing this strand from the complex (Rand et al., 2005). Once the mRNA has been cleaved, and due to the presence of unprotected RNA ends in the fragments, the mRNA is further cleaved and degraded by intracellular nucleases and will no longer be translated into protein (Orban & Izaurralde, 2005) while RISC will be recycled for subsequent rounds (Hutvagner & Zamore, 2002). This constitutes a catalytic process leading to the selective reduction of specific mRNA molecules and of the corresponding proteins. It is possible to exploit this native mechanism for gene silencing with the purpose of regulating any gene(s) of choice by directly delivering siRNA effectors into the cells or tissues, where they will activate RISC and produce a potent and specific silencing of the targeted mRNA.

PTGS cannot only be induced by siRNA through sequence specific cleavage of perfectly complementary mRNA. Recent discoveries have reported the existence of other endogenous post-transcriptional regulatory mechanisms. One of these mechanisms is that mediated by miRNAs, which are functional naturally occurring small non coding RNAs only require partially complementary targets to bind to their target mRNAs through their 3′ untranslated regions (3′ UTRs) (Lee et al., 1993; Wightman et al., 1993). miRNAs act as guide sequences to

regulate the expression of multiple genes that are often functionally related. Furthermore, the translation of many mRNAs is regulated by multiple different miRNAs. They are critical factors in coordinating the development, differentiation and function of cells and tissues and it is estimated there are hundreds of these molecules in humans. There are approximately 1400 miRNAs that have been identified in the human genome and they are believed to regulate the expression of up to 30% of all human genes by preventing translation of mRNAs into proteins. For further details on miRNA biology and therapeutic potential refer to section 6.

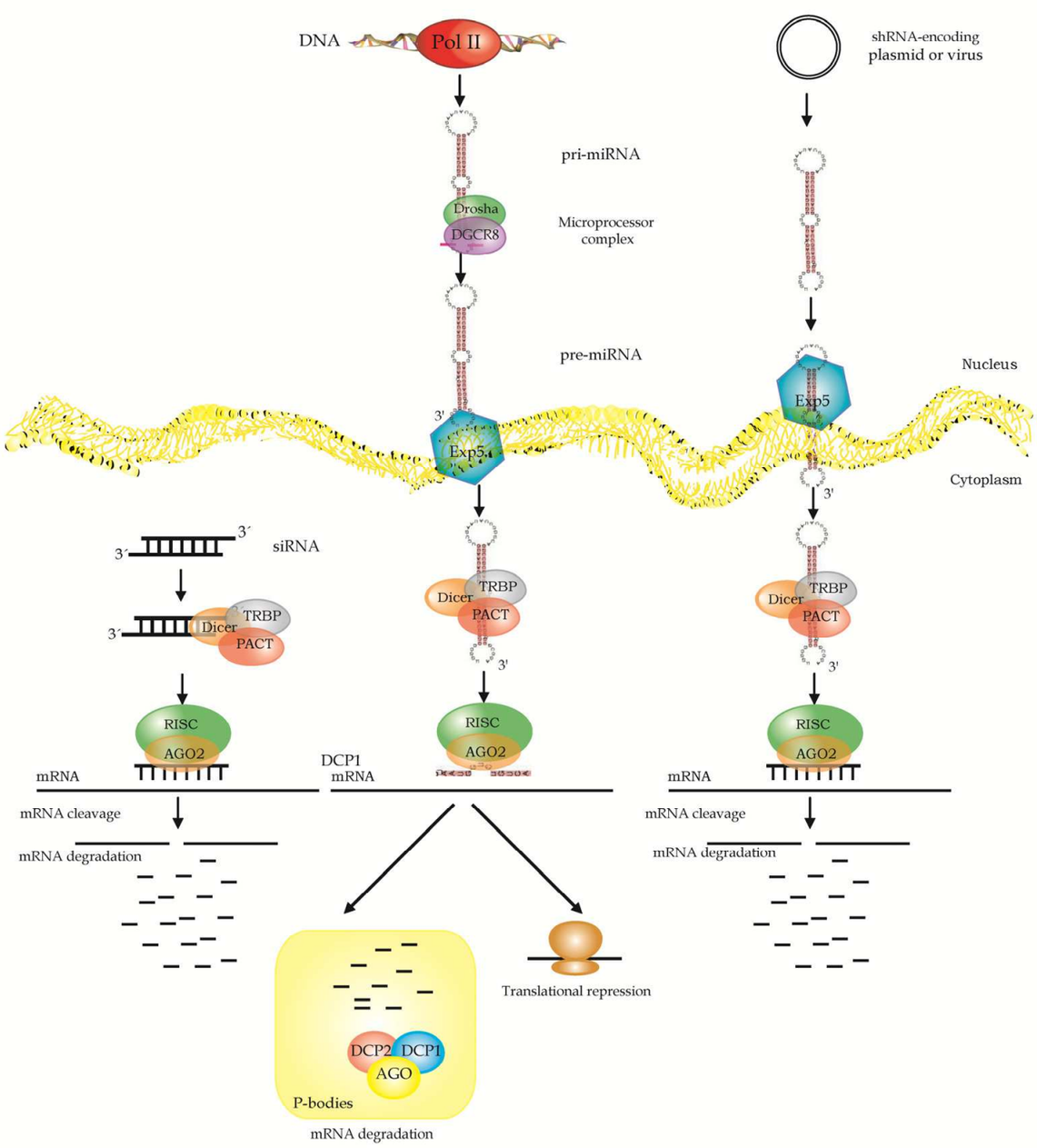

Fig. 1. Mechanism of RNA interference in mammalian cells. RNA interference (RNAi) is an intracellular mechanism triggered through small RNAs that include small interfering RNAs

(siRNAs), microRNAs (miRNAs) and short hairpin RNAs (shRNAs). The siRNA pathway begins when double stranded RNAs (dsRNAs) are trimmed down by the Dicer complex into siRNAs. Alternatively, synthetic siRNAs can be introduced directly into the cell cytoplasm. These siRNAs are incorporated into the RNA-induced silencing complex (RISC), where they are unwound. If the siRNA has perfect sequence complementarity, the Argonaute 2 protein (AGO2) present in RISC cleaves the passenger (sense) strand so that active RISC containing the guide (antisense) strand can recognize target sites on the messenger RNA (mRNA) to direct mRNA cleavage. This cleavage is performed by the catalytic domain of AGO2. The miRNA pathway starts when primary miRNA (pri-miRNAs) are transcribed from RNA polymerase II (Pol II) promoters, forming hairpin-shaped structures. These are processed by the Drosha-containing microprocessor complex, giving rise to precursor miRNAs (pre-miRNAs), that are also stem-like structures with a 2-nucleotide 3′ overhang. Pre-miRNAs are transported into the cytoplasm by Exportin 5 (Exp5), where they are processed by a Dicer containing complex to ~21-25 nucleotide imperfect dsRNA duplexes that constitute the mature miRNAs. Once the miRNA duplex is processed, the guide sequence is loaded into RISC and then mediates binding to the target sequence in the 3′ UTR of cellular mRNAs. If the miRNA guide sequence is fully complementary to its target site, it triggers site-specific cleavage and degradation of the mRNA through the catalytic domain of AGO2. On the other hand, if the base pairing is incomplete but fully complementary in the seed region (nucleotides 2-8 of the miRNA), repression of protein expression occurs, often accompanied by mRNA degradation in cytoplasmic processing (P)-bodies. Mimicking the miRNA mechanism, synthetic DNA vector constructs or viral particles code for stable shRNAs, that are transcribed from a RNA polymerase II/III promoter and form haipin-like structures. These shRNAs are transported into the cytoplasm by Exp5 and recognized by Dicer, leading to the formation of siRNAs homologous to the target mRNA and, subsequently, to mRNA degradation.

Commercially available systems and other therapeutic initiatives aimed at mimicking the mechanism of RNAi make use of DNA vector constructs or viral particles coding for long term and stable short hairpin (shRNAs) expression that are transcribed from a RNA polymerase III promoter *in vivo* or shRNAs that are synthesized exogenously and transfected into the cells. The double stranded region of shRNAs is formed though a hairpin structure and intramolecular hybridization that resembles that of miRNA precursors (Brummelkamp et al., 2002a; Paddison et al., 2002) (Figure 1). These shRNAs molecules are recognized by Dicer, leading to the formation of siRNAs homologous to the target mRNA. The main difference with siRNAs is that while these mediate only transient silencing, virally expressed shRNAs mediate a very potent and stable silencing effect for as long as their transcription takes place. shRNAs also enter the endogenous silencing pathway at an earlier stage than siRNAs, having a higher chance of saturating the natural miRNA natural pathways (Grimm & Kay, 2006).

3. Efficacy and stability

3.1 Basic design rules

Bioinformatic tools play an important role in RNAi design. Several tools are available for this purpose and many papers have been published that describe algorithms for selecting RNA sites. Algorithms have been developed that can help select siRNA target sites that exhibit high target-specific activity and minimal off-target activity. Efficacy of siRNAs for

individual targets normally depends on different factors, such as thermodynamic stability (Khvorova et al., 2003), structural features (Castanotto et al., 2007), target mRNA accessibility (Patzel et al., 2005) and additional position specific determinants (Heale et al., 2005; Schwarz et al., 2003). Systematic studies on targeting efficacies have shown that optimal siRNAs should be between 19 and 25 nucleotides long, should have 3' symmetric dinucleotide overhangs, low guanine-cytosine content (between 30 and 52%) (Schwarz, et al., 2003) and specific nucleotides at certain positions. For example, features that increase siRNA efficacy are the presence of an adenine or uracil in position 1, adenosine in position 3, a uracil in positions 7 and 11, a guanine in position 13, a uracil or adenine in position 10 (this is the site for RISC mediated cleavage), a guanine in position 21 and/or the absence of guanines or cytosine at position 19 of the sense strand (see (Amarzguioui & Prydz, 2004) for a full review of the topic). In general, enrichment in adenosines and uracils along the first 6-7 base pairs of the sequence, and consequently, weak hydrogen bonding, allows RISC to easily unravel the double stranded duplex and load the guide strand (Dykxhoorn & Lieberman, 2006).

As mentioned above, the siRNA duplex should also be thermodynamically flexible at its 3' end, i.e., at positions 15-19 of the sense strand. This correlates with its silencing efficacy such that the presence of at least one adenosine-uracil pair in this region would decrease internal stability and increase silencing efficacy. On the contrary, internal repeats or palindrome sequences decrease the silencing potential of siRNAs.

Another consideration to be taken into account when designing an siRNA sequence is the nature of the target sequence. Under certain circumstances it will be preferable to include all splice variants and isoforms for the design of siRNAs whereas, in other instances, they should be specifically left out. Similarly, attention should be paid in order to choose sequences within the coding region of the target gene sequence, as gene silencing is an exclusively cytoplasmic process (Pei & Tuschl, 2006). Good news is that, taking all these established criteria into account, RNAi allows for an almost unrestricted choice of targets. Nevertheless, any theoretically optimal siRNA will require extensive testing to achieve high silencing efficacy without any unwanted side effects.

3.2 Fate of RNAi in biological fluids

Conventional siRNA is degraded within minutes in a serum-containing environment, which hampers their *in vivo* use. Depending on which delivery method is selected, stabilizing RNAi compounds by chemical modification may be critical for RNAi activity.

Various modifications improve siRNA stability in biological tissues and fluids. It is possible to modify siRNA molecules chemically without significant loss of activity. However, a fundamental requirement of siRNA function is that the antisense strand must either have a free hydroxyl or a phosphate at the 5'-terminus and therefore, this terminus cannot be modified. Of all the internal modifications, substitutions of the 2'OH of ribose remain the best studied and include –H,-OMe and –F. Positions 9 and 10 on the sense strand of the siRNA duplex are particularly sensitive to chemical modification, as cleavage at this position is necessary for removal of the sense strand and activation of RISC (Braasch et al., 2003; Martinez et al., 2002).

Locked Nucleic Acids (LNAs) are a family of conformationally nucleotide analogs, which provides very high affinity and high nuclease resistance to DNA and RNA oligonucleotides. This type of modification is compatible with siRNA machinery and has been reported to increase potency with minimal toxicity. Conjugating siRNA to large molecules, or

incorporating them into liposomes or nanoparticles, has been used to improve the pharmacokinetic properties of siRNAs by increasing the stability of the molecule to nuclease degradation and slowing the rate of renal clearance for small RNAs.

3.3 Tissue penetrance, intracellular delivery and targeting specific cells and/or tissues

One of the major problems in the development of RNAi-based therapies is the delivery of these molecules to the desired target cells, within their corresponding tissues and organs. The high therapeutic potential of RNAi compounds and their application in clinical settings is currently limited due to the lack of efficient delivery systems. For safe and effective delivery of RNAi to the mRNA target, many variables must be negotiated, e.g. size and diameter of delivery particles, toxicity, clearance of particle components and targeting to the appropriate points of action will be discussed.

Viral siRNA delivery has been used to specifically down-regulate the expression of genes of pathological relevance, especially for chronic diseases in which long-term gene silencing is desired, e.g. neurodegenerative disorders, cancer, heart failure and Human Immunodeficiency Virus (HIV) infections. This is achieved using gene therapy approaches in which an shRNA expression cassette is stably integrated into the host cell genome or expressed episomally. Subsequent sections of this chapter deal with specific developments based on expressed RNAi, the most notable being a treatment for HIV infection which has reached the clinic. Viral delivery systems have the advantage of achieving high transfection efficiencies due to the inherent ability of viruses to transport genetic material into cells. However, viral systems have a limited loading capacity, and pose severe safety risks because of their oncogenic potential via insertional mutagenesis (Lehrman, 1999; Sinn et al., 2005), their inflammatory and immunogenic effects (Donahue et al., 1992; Liu & Muruve, 2003) and the difficulties in controlling the timing and dose of interference. Therefore it is necessary to develop improved viral vectors that could target specific cell types or tissues after systemic *in vivo* administration in order to minimize toxicities associated with treatment.

Due to these diverse safety issues, non-viral delivery strategies have been extensively researched and more widely used. However, the issue of RNAi compound delivery has not yet been solved to a degree that allows their widespread use in therapy.

siRNAs are generally not taken up by mammalian cells, including those that actively sample their environment. However, certain tissues and cells in the lungs, mucosal environments, eyes, and even the central nervous system have been shown to efficiently take up siRNAs in the absence of transfection reagents (Bitko & Barik, 2007; Bitko et al., 2005; Luo et al., 2005; Nonobe et al., 2009; Thakker et al., 2004). As will be discussed later, some developments based on local delivery have reached the clinic, including those related to ocular, skin and lung diseases. Nevertheless, since uptake of naked siRNAs is not always possible, different options have been approached, such as bioconjugation and complex formation.

Bioconjugation strategies include conjugation with lipids, which may enhance siRNA uptake via receptor-mediated endocytosis or by increased membrane permeability of the negatively charged RNA. Although α-tocopherol conjugation to siRNA has also been described (Jeong et al., 2009), the most extensively used bioconjugate lipid has been cholesterol, that has proved to induce intracellular RNAi without any significant loss of gene silencing activity when compared to the unconjugated version, and with a good hepatic deposition after systemic administration *in vivo* (Cheng et al., 2006; Lorenz et al.,

2004; Soutschek et al., 2004). Cell penetrating peptides (CPPs) have also been proposed as an alternative to traditional methods of siRNA delivery. CPPs are short amino acid sequences, consisting mainly of positively charged amino acids that are able to interact with the plasma membrane, leading to a highly efficient uptake into the cytoplasm. Remarkable enhanced uptake effects have been shown by Davidson and coworkers in primary neuronal cells using a penetratin-coupled siRNA against several endogenous proteins (Davidson et al., 2004). siRNAs have also been conjugated to polyethylene glycol (PEG) showing increased resistance to serum degradation (Kim et al., 2006). These conjugates have also been further complexed with additional condensing agents to form colloidal nanoparticles (Jeong, et al., 2009). However, to date, none of these bioconjugates have reached the clinic without further encapsulation.

Regarding complex formation, several alternative developments have provided interesting results, some even having reached Phase I clinical trials, as discussed later on.

The flexibility in the design of cationic lipid structures and liposome composition, together with their *in vivo* efficiency, have promoted the notion that cationic lipids can be efficiently used for human gene transfer. Nucleic acids, including siRNAs, are able to electrostatically interact with cationic liposome-forming particles. However, due to their small size, they cannot condense into particles of nanometric dimensions (Spagnou et al., 2004). A development based on cationic lipids are stable nucleic acid lipid particles (SNALPs), which were developed by Tekmira Pharmaceuticals and have reached clinical trials for the treatment of hypercholesterolemia.

As with liposomes, the charged nature of siRNAs allows their complexation with various cationic polymers based on electrostatic interactions. Polymers used for delivery can be divided into two main categories: (i) those of synthetic origin, such as dendrimers, polyethyleneimine, and poly-L-Lysine; (ii) those of natural origin that are biodegradable and more easily degraded and excreted from the body, such as atelocollagen, gelatine, chitosan and cyclodextrin (Vorhies & Nemunaitis, 2009).

One of the most striking delivery methods, which has also reached clinical testing, was initially developed by Cequent Pharmaceuticals and was named transkingdom RNAi (tkRNAi). This technology uses non-pathogenic *E. coli* bacteria to produce and deliver therapeutic shRNA- into target cells to induce RNAi (Kruhn et al., 2009).

4. Safety issues related to the therapeutic use of RNAi technologies

4.1 Immune-mediated toxicities

The immune system has evolved cellular and molecular strategies to discriminate between foreign and self nucleic acids. It is activated by microbial RNA and DNA, leading to the production of type I interferon (IFN) and proinflammatory cytokines. Among the cytoplasmic sensors of long dsRNA is the dsRNA-dependent protein kinase (PKR), that phosphorylates translation initiation factor IF-2α leading to translation arrest, inhibition of protein synthesis and induction of apoptosis. This mechanism is an essential step in antiviral resistance (Akira & Takeda, 2004; Peters et al., 2006). Most human cells constitutively express low levels of PKR that remain inactive. Upon binding to a 30-80 nucleotide dsRNA fragment in a sequence-independent manner, PKR forms a homodimer, leading to its phosphorylation and activation. PKR can also activate the NF-κB signalling pathway via the phosphorylation of IKKβ (Sioud, 2010). A second protein that is stimulated by dsRNA is 2'-

5′ oligoadenylate synthetase (OAS), which is expressed constitutively and also upregulated by type I IFNs during antiviral responses (Samuel, 2001). This IFN–induced enzyme catalyzes the formation of 2′-5′-linked oligoadenylates from ATP that activate a latent ribonuclease, called RNAse L, that degrades both cellular and viral RNAs (Sioud, 2010). Although both OAS and PKR are involved in antiviral immunity, PKR and RNAse L are mainly IFN effectors and not absolutely required for IFN production. Therefore, other kinases may be involved. Two additional factors, intracellular cytosolic DExD/H box RNA-helicases retinoic-acid–inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5), have been identified as cytoplasmic sensors of viral dsRNA (Kato et al., 2006). Although RIG-I seems to be an important sensor of viral RNAs, microbial nucleic acids are also recognized by Toll Like Receptors (TLRs) , especially in immune cells (Sioud, 2006), making them crucial in sensing viral and bacterial nucleic acids. Whereas most TLRs are expressed on the plasma membrane, detecting extracellular bacterial components, TLR3, TLR7 , TLR8 and TLR9 are expressed in intracellular compartments (endosomes and lysosomes) (Takeda & Akira, 2005), allowing them to sense viral RNAs. While TLR3 is expressed on the cell surface and is believed to recognize viral dsRNA released during cell lysis (Alexopoulou et al., 2001), TLR4 and TLR8 recognize viral single-stranded RNA (ssRNA) (Heil et al., 2004). Recent studies have demonstrated how PKR and TLR3 are not the major pathways by which chemically synthesized siRNAs activate inmmunity (Hornung et al., 2005; Judge et al., 2006; Sioud, 2006). Indeed, certain siRNA sequences stimulate monocytes via TLR8 or dendritic cells via TLR7 to produce proinflammatory cytokines and IFN α (Sioud, 2005). Sometimes TLR7/8 induction is also sequence-dependent, as they recognize certain siRNA sequence motifs, such as 5′-UGUGU-3′ (Judge et al., 2005) and 5′-GUCCUUCAA-3′ (Hornung, et al., 2005). Many other sequences with high uridine content can also activate immune responses (Sioud, 2006).

In order to overcome siRNA immune activation siRNA design can be improved in many ways, such as:

- Avoiding sequence motifs and high uridine content
- By introducing modifications like 2′-deoxy. 2′-O-methyl, 2′-fluoro or selected LNA modifications in nucleotides and/or their backbone can abrogate immune response without compromising efficiency and silencing potency.(Hornung, et al., 2005; Judge, et al., 2006; Morrissey et al., 2005b; Sioud, 2006, 2010)
- By using naturally modified nucleotides such as 5′-methylcytidine, 5-methyluracil, N6-methyladenosine, 2-thiouridine or pseudo-uridine (Kariko et al., 2005)
- Staving off the activation of RIG-I, that recognises blunt end dsRNAs, by adding 2 nucleotide 3′-overhangs in the siRNA structure. These overhangs allow the siRNAs to escape recognition by mimicking endogenous small RNAs processed by Dicer (Marques et al., 2006)
- Using antibody-mediated receptor specific delivery techniques (Song et al., 2005) and agents, such as chloroquine or bafilomycin, which impede the activation of TLR7/8 by inhibiting siRNA retention in the endosomes (Sioud, 2005).

4.2 Non immune off target effects

The design of siRNAs includes the selection of siRNA sequences that are capable of knocking down the expression of their target genes. The cleavage of intended target RNAs by AGO2-RISC is highly sequence-specific and only a few mistmaches between the guide

strand and the target are tolerated (Dahlgren et al., 2008). Yet, siRNAs can trigger unintended silencing of hundreds of endogenous genes, resulting in toxic phenotypes and compromising the interpretation and outcome of the particular siRNA application (Birmingham et al., 2006). These siRNA off-target effects (OTEs) are primarily mediated by the interaction between the seed region of the RISC-associated guide strand (nucleotides 2 to 8 from the 5′ end) and complementary sites in the 3′UTR of the mRNA (Birmingham, et al., 2006; Jackson & Linsley, 2009; Jackson et al., 2006; Lin et al., 2005). Upon target binding, even partially complementary off targets are silenced through several mechanisms such as translational inhibition and mRNA destabilization (Doench & Sharp, 2004; Wu et al., 2006). Careful comparison of candidate guide strand sequence with the entire transcriptome, attempting to avoid long stretches of homology, might reduce the risk but it has been estimated that approximately 83% of the possible 21 mers within the coding sequences of the genome are unique, leaving one out of every five 21 nucleotide long siRNAs to display some homology compared with a given mRNA. Snove and Holen performed an independent investigation of 360 published siRNA sequences and found that almost 75% of the analysed oligonucleotides had the potential to trigger unwanted OTEs. They suggested that the use of inappropriate programs, such as basic local alignment (BLAST), to design silencing oligonucleotides lead to abundant OTEs because a precise homologous stretch of six or seven base pairs is necessary for detection of homology through BLAST (Snove & Holen, 2004).

The specificity of an siRNA sequence can be improved by taking into account some important designing parameters, such as thermodynamic stability of the duplex at the 5′ and 3′ ends, the Tm value of the seed sequence region (Ui-Tei et al., 2004) and the selection of the target position trying to avoid regions very close to the initiation codon (Yuan et al., 2004). As the siRNA silencing effect is concentration-dependent, success in reducing siRNA OTEs can be achieved by optimizing doses and using siRNA pools in order to minimize the contribution of individual siRNAs while preserving on-target activity (Bramsen & Kjems, 2011). An improvement on specificity could be also achieved by altering siRNA sequences and/or by introducing chemical modifications that are able to reduce off-target potentials. Several studies have aimed at reducing siRNA OTEs by chemically modifying the seed region of the guide strand with 2′O Me on position 2 (Jackson, et al., 2006) or by incorporating a strongly destabilizing unlocked nucleic acid (UNA) modification at position 7, which induces a position-specific destabilization of seed-target interactions (Bramsen & Kjems, 2011). Similarly, replacing of seed sequence nucleotides by deoxynucleotides results in a reduction of OTEs (Ui-Tei et al., 2008). These modifications avoid incorporation of the sense strand and promote incorporation of the antisense strand of the siRNA duplex into the RISC complex. Finally, another way of reducing the unwanted effects is by striving for very specific delivery, i.e., the more targeted the delivery of the siRNAs, the less the likelihood of suffering OTEs.

4.3 Oversaturation of endogenous RNAi-silencing complex

Bioactive drugs that rely on cellular processes to exert their functions face the risk of saturating endogenous pathways. This may be the case with RNAi-based drugs. Naturally occurring small RNAs exist in a perfect balance with their precursors and targets, as well as with the associated machinery involved in this process. Gene silencing is performed by

introducing artificially synthesized small RNAs into the cell or by inserting siRNAs/shRNAs within the cell, which enter the endogenous RNAi pathway at different levels. shRNAs and siRNAs are very similar to miRNA precursors before and after Dicer processing, respectively, relying on endogenous miRNA machinery to achieve target silencing. Therefore, miRNA pathways might get saturated by high doses of exogenous RNAs. One of the ways adenovirus avoid potential host RNAi antiviral activity is by expressing high amounts of a non-coding RNA stem-loop that interferes with transport from the nucleus to the cytoplasm by binding to the nuclear karyopherin Exp-5, inhibiting transport and subsequent processing of cellular pre-miRNAs (Fedorov et al., 2005). Similarly, some reports have described that *in vivo* adeno-associated virus (AAV)-encoded overexpression of liver-directed shRNAs can saturate Exp-5. This results in the inhibition of endogenous pre-miRNA nuclear export and, ultimately, death (Grimm et al., 2006). Strong expression of shRNAs has also been shown to induce cytotoxicity in primary lymphocytes, whereas the same shRNA expressed using a weaker promoter presents no toxic effects (Lu & Cullen, 2004). Similarly, robust levels of antisense RNAs emerging from shRNA expression systems cause toxicity in the mouse brain, regardless of the sequence (An et al., 2006). The export function mediated by Exp-5 is not required for the activity of synthetic siRNAs (McBride et al., 2008) but a recent report has shown that synthetic siRNAs and expressed shRNAs compete against each other and with endogenous miRNAs for transport and incorporation into RISC and that TRBP is one of the sensors for selection and incorporation of the guide sequence of interfering RNAs (Yi et al., 2003). If the siRNA design parameters are not optimal they might cause imbalance of the endogenous small RNA mediated pathways resulting in various and deleterious unwanted effects in the cells. Thus, a number of factors altering endogenous cellular processes can result in toxicity. It becomes crucial to optimize the siRNA/shRNA design parameters and work at the lowest possible concentrations to mitigate the potential of unwanted side effects.

5. Targets addressable by RNAi: Therapeutic application of RNAi

Since the first description of RNAi in 1998 by Tuschl and coworkers (Tuschl et al., 1998), this mechanism has rapidly been exploited for therapeutic applications. To date, several RNAi-based drugs against a variety of targets in humans have been developed. Antisense strategies offer very high target specificity, having the potential to lead a revolution in the field of drug development. Additionally, the relatively short turnaround for efficacy testing of potential RNAi molecules and the fact that any target is theoretically amenable to targeting, makes them invaluable tools to treat a wide range of diseases.

5.1 Infectious diseases

5.1.1 Virus infections

Viral infections are usually difficult to treat with conventional drugs and, in the cases where success is achieved, drug resistance may rapidly become an issue. This latter characteristic, together with the fact that viral genes are substantially different from human genes, makes viral infections obvious candidates for RNAi therapy. In order to obtain durable and effective antiviral therapies, viral proteins that can be disabled need to be identified. Ideally, these targets should be essential factors that share conserved sequences across many

different strains or even among different species of virus of the same family, so that a single target will have broad effectiveness (Das et al., 2004; Wilson & Richardson, 2005). In addition, target sequences should be as different as possible from any human protein to reduce the likelihood of side effects. Alternatively, host factors essential for virus replication could also be targeted, reducing the risk of viral escape, although the chances of affecting vital cell processes by this approach are considerable. The main caveat of targeting viral infection with RNAi is the ability of most viruses to mutate their target sequences in order to escape RNAi attack.

Several reports have indicated the promise of siRNAs for treatment of viral infections, showing a cessation of viral proliferation in human cell cultures. Upon exposure to siRNA, cells shut down production of the proteins needed for pathogen reproduction. Research is ongoing to find antiviral compounds for the following viral infections: hepatitis C and B virus (McCaffrey et al., 2003; Morrissey et al., 2005a), HIV type 1 (DiGiusto et al., 2010), human papillomavirus type (HPV) 16 (Jung et al., 2010; Palanichamy et al., 2010), influenza viruses A (Seth et al., 2010; Zhiqiang et al., 2010), respiratory syncytial virus (RSV) (Bitko & Barik, 2001; Bitko, et al., 2005; DeVincenzo, J. et al., 2010; DeVincenzo, J.P. et al., 2010; Zamora et al., 2010), herpes simplex virus 2 (Palliser et al., 2006), West Nile virus (WNV) (Anthony et al., 2009; Ye et al., 2011), severe acute respiratory syndrome (SARS) virus (Li et al., 2005; Wu & Chan, 2006) and cytomegalovirus (CMV) (Wiebusch et al., 2004).

Sereval R&D programs are currently focusing their efforts on infection diseases, HIV is one of these examples. Introduction of synthetic siRNA into cells or its stable endogenous production using vector-driven shRNA have been shown to suppress HIV replication *in vitro* and, in some instances, *in vivo* (Subramanya et al., 2010). RNAi can specifically degrade the HIV-1 genome in infected T cells very early in the viral replication cycle. This suggests the possibility of a therapeutic strategy that targets the virus before it has a chance to develop escape mutations. To date numerous siRNAs targeted to a number of HIV-1 or host dependency factors (HDF) transcripts have been demonstrated to achieve viral inhibition both *in vitro* and *in vivo*. Indeed, HIV-1 encoded genes tat, rev, gag, pol, nef, vif, env, vpr and the long terminal repeat (LTR) are all susceptible to RNAi–induced gene silencing in cell lines (Tsygankov, 2009). Several researchers have described the ability of siRNAs to interfere with HIV viral production after infection before mutations can occur. However, after a provirus is established, many thousands of viral transcripts are generated *de novo* in the infected cell and degradation of these is a far greater task for the RNAi machinery. For the development of a durable gene therapy that prevents viral escape, a multiple shRNA approach has been proposed against conserved HIV-1 regions (Schopman et al., 2010; ter Brake et al., 2006). Expression of three different shRNAs from a single lentiviral vector resulted in high levels of inhibition. Thus, their combined expression induced a much stronger inhibition of virus production.

Scientists at Benitec Ltd in collaboration with the City of Hope Medical Center in California are investigating a method to fight HIV infection in lymphoma patients with genetically modified stem cells, eradicating both the lymphoma and the HIV infection at the same time. The treatment is the first to use specially engineered anti-HIV genes inserted into the patients' own harvested stem cells. The cells are then reinfused into the HIV-infected patient. If successful, the new treatment would allow the organism of the patient to produce HIV-resistant white blood cells indefinitely. A triple combination lentiviral construct is used

to carry the gene segments into the stem cells. The lentivirus vector encodes 3 forms of anti-HIV RNA: (1) RNAi as shRNA targeted to HIV-1; (2) a decoy for the HIV TAT-reactive element (TAR); and (3) a ribozyme that targets the host cell CCR5 chemokine receptor. This compound received FDA approval and entered phase I clinical trial in 2007 (DiGiusto, et al., 2010).

Several well-known HDF, including NFkappaB, CD4 receptor and coreceptors CCR5 and CXCR4, have been successfully targeted by siRNAs, thereby suppressing HIV replication or entry (Singh & Gaur, 2009). A potentially promising strategy is to exploit siRNAs to prevent viral entry at the cell surface by downregulating essential cell surface HIV-1 co-receptors. This approach has been used by two groups, the first one targeted the CXCR4 co-receptor with siRNA (Anderson et al., 2003); while the second group transduced T lymphocytes with a CC5-siRNA reducing cell membrane CCR5, and protecting the cells from infection with R5-tropic HIV (Cordelier et al., 2003). Transduction with CCR5-siRNAs substantially reduced CCR5 mRNA in other cell lines such as CCR5 + cell lines, primary human macrophages and brain microglia. Scientists at the California Institute of Technology have reported success in constructing a lentivirus-based vector to introduce siRNAs against the HIV-1 coreceptor CCR5 into human peripheral blood T lymphocytes (Qin et al., 2003). This approach has not only successfully reduced CCR5 expression but also provided substantial protection from CCR5-tropic HIV-1 virus infection, dropping infected cells by 3- to 7-fold. Also bi-specific siRNA constructs, containing an 8 nucleotide intervening spacer targeted against CXCR4 and CD4 or CXCR4 and CCR5 have been shown to inhibit viral entry (Anderson & Akkina, 2005). Cleavage of the bispecific constructs yielding monospecific siRNAs was shown to occur in cell extracts showing significant downregulation of their respective coreceptors. These results demonstrated the practical utility of short hairpin shRNA bi-specific constructs synthesized as single transcripts. It is now possible to introduce promising multivalent shRNA constructs into retroviral and lentiviral vectors for *in vivo* gene therapeutic applications.

5.1.2 Parasitic diseases

Parasitic infections are especially endemic in low-income populations in developing regions of Africa, America and Asia. They include, among others, schistosomiasis infections, leishmaniasis, leprosy, tuberculosis and malaria. Although a few initial approaches have suggested RNAi may be a useful tool in the treatment of some of the neglected tropical diseases, it is the treatment of malaria that has received the most attention.

Despite intense efforts, malaria remains a leading cause of morbidity and mortality worldwide. Recent evidence strongly suggests that RNAi can play a key role in identifying the genetic factors that shape the vector parasite relationship and may be crucial to identifying new genetic means of controlling mosquito-borne diseases (Vlachou et al., 2005). The Institute of Molecular Medicine (IMM) in Lisbon has pioneered an *in vitro* assay system to monitor the crucial process of sporozoite infection using the malaria parasite *Plasmodium berghei* with cultured human liver cells. Cenix BioScience has collaborated with IMM to adapt and optimize the malarial infection assay for high throughput kinome-wide RNAi screening. The screen led to discovery of five targets clearly implicated in malaria, all of which resulted in significant reductions in infection *in vitro* when silenced with small interfering siRNAs (Prudencio et al., 2008). PKCζ, was selected for further evaluation *in vivo*. In mice given a systemically delivered, liposomally formulated anti-PKCζ RNAi

therapeutic, an inhibitory effect on infection was observed. In fact, loss of PKCζ function *in vivo* by RNAi-mediated silencing led to an 80% decreased infection rate. In addition, certain siRNAs also led to a delay in the appearance of parasites in blood and a significant reduction in average blood parasitemia. The identified genes will represent excellent candidates for the development of novel antimalarial therapeutics.

5.2 Neurological disorders

Neurodegenerative disorders are a group of neurological disorders characterized by selective dysfunction and eventual death of distinct subpopulations of neurons in the central nervous system, accompanied by a concomitant decline in specific neurological functions. Currently there are no effective therapies for most neurodegenerative diseases (DeKosky & Marek, 2003; Shaw et al., 2007). Whenever treatments are available, such as in the case of Alzheimer's disease (AD) or Parkinson's diseases (PD), these treatments provide only partial relief for some of the symptoms. In recent years, significant research effort has focused on understanding the underlying pathologies of diseases such as AD, PD, Huntington disease (HD) and amyotrophic lateral sclerosis (ALS).

The key therapeutic advantage of using RNAi for the treatment of neurodegenerative diseases is its ability to specifically and potently knock-down the expression of disease-causing genes of known sequence. In several neurodegenerative diseases, such as prion mediated diseases (Schwartz, 2009; White et al., 2008), polyQ-repeat diseases (HD and spinocerebellar ataxias) (Schwartz, 2009; Seyhan, 2011) PD and ALS (Maxwell, 2009), a single allele is mutated. This mutated allele leads to the production of a toxic protein, causative agent of the pathology.

AD is an example of neurological disorder addressable by RNAi technology. Tau and amyloid precursor protein (APP) are involved in the pathogenesis of sporadic and inherited AD. Certain dominantly inherited mutations have been associated with early onset forms of AD. Scientists at the University of Iowa have developed siRNAs that display optimal allele-specific silencing against a well-characterized Tau mutation (V337M) and the most widely studied APP mutation (APPsw) (Miller et al., 2004). Recent studies have shown that a reduction in BACE-1 expression using siRNA or lentiviral vectors expressing siRNAs targeting *BACE-1,* results in reduced Aβ production in primary neurons from wild-type and APPsw transgenic mice, showing a reduction in the number and size of plaques and protection from oxidative stress (Kao et al., 2004; Singer et al., 2005). Other potential therapeutic targets for siRNA-mediated gene silencing in AD are α- and β-secretases.

ALS is a progressive, devastating syndrome that affects both upper and motor neurons. ALS involves a multifactorial and interactive chain of pathogenic mechanisms that are probably common to several neurodegenerative disorders. Various therapies have been tried but none has proved to stop progression of the disease. In 25% of familial ALS cases, the disease is caused by dominantly acting point mutations in the gene superoxide dismutase (SOD1). RNAi has been evaluated for selective silencing of mutant SOD1 expression in cultured cells. In one study, siRNAs were capable of specifically inhibiting expression of ALS-linked mutant, but not wild-type SOD1, protecting from induced cell death (Maxwell et al., 2004). The main challenge of creating a potential RNAi-based therapeutic for ALS is in the large number of different SOD mutations that have been identified. In inducible mouse models of spinocerebellar ataxia type 1 (SCA1) and HD, repression of the mutant allele

expression improves disease phenotypes. Thus, therapies designed to inhibit expression of the mutant gene should be beneficial. Scientists at the University of Iowa College of Medicine in collaboration with the University of Minnesota and the NIH have shown that RNAi can inhibit polyglutamine-induced neurodegeneration caused by mutant ataxin-1 in a mouse model of SCA1. Mice suffering from SCA1 treated with RNAi-based compounds had normal movement and coordination, and their brain cells were protected from the destruction normally caused by the disease.

HD gene has been cloned and the mutation contains an unstable trinucleotide repeat (CAG) that expands the length of a repeated stretch of amino acid glutamine in the gene product, a protein called huntingtin. Currently there is no cure for HD and treatments can only alleviate disease symptoms (Harper, 2009) RNAi directed against mutant human *hungtingtin* reduces hungtingtin mRNA and huntingtin protein expression in cell culture and in the brain of a HD mouse model after intrastriatal treatment with AVV-expressing shRNA against human huntingtin (Harper et al., 2005). Detailed examination of the protein levels in the treated mice showed that levels of the toxic protein were reduced to about 40% of the levels found in untreated mice. Behavioural and neuropathological abnormalities associated with HD improved following *hungtingtin* gene silencing.

5.3 Respiratory diseases

Targeted, local delivery of RNAi to the lungs via inhalation offers a unique opportunity to treat a wide range of untreatable respiratory conditions. Delivering the drug to the lungs by instillation allows for direct access to the lung epithelial cells. This direct targeting to the lung cells allows for a reduction in the dosage of the siRNA required for achieving efficacy and simultaneously diminishes the likelihood of off target effects. Diseases treatable with this approach include viral lung diseases, cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), allergy, asthma and lung cancer among others.

CF is the most common autosomal recessive disorder in Caucasians. A defective gene causes the body to produce abnormally thick, sticky mucus that obstructs the lungs, leading to life-threatening lung infections, and the pancreas, causing difficulty absorbing food. This genetic disease is marked by defects in a protein, known as the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein, required for proper transport of salt and water across the cell membrane. It has been hypothesized that the missing function of CTFR causes an increased ENaC-mediated sodium uptake from the luminal secretion of the airways, deplets the airway surface liquid and leads to defective mucociliary clearance (Chambers et al., 2007) and to the production of a thick and sticky mucus that clogs the lungs and the digestive system. This promotes bacterial infections, resulting in a slow progressive destruction of the airway tissue and difficulties in absorbing food. Due to the central role of ENaC-mediated sodium uptake by the respiratory epithelium for the pathogenesis of CF, it has been postulated that a reduction in ENaC activity could attenuate CF lung disease (Yueksekdag et al., 2010). Yueksekdag and co-workers have designed, synthesized and first used ENaC-specific small interfering RNAs in M-1 cell cultures and in a mouse model (Yueksekdag, et al., 2010). Their results showed that a single *in vitro* application was sufficient to decrease ENaC mRNA levels, whereas repeated administrations were necessary to obtain a significant reduction for ENaC mRNA level in the *in vivo* application.

Asthma is marked by inflammation of the airways and Syk kinase has been described to play a critical role in the regulation of such immune and inflammatory responses. Initial

studies used aerolised Syk-specific antisense oligonucleotides in liposome complexes to significantly decrease lung inflammatory responses in asthma and acute lung injury models (Ulanova et al., 2005). This approach has been further developed by Canadian biotech Zabecor, who is developing an siRNA against Syk for asthma treatment. It has also been suggested that IL-5 is involved in the development of airway hyper-responsiveness (AHR). siRNAs targeting IL-5 were characterized *in vitro*, and administered intratracheally to OVA-induced murine model of asthma (Huang et al., 2008). siRNA targeting IL-5, efficiently moderated the characteristics of asthma, including AHR, cellular infiltration of lung tissues, and IL-5 mRNA levels in lungs in the mouse model of asthma.

5.4 Skin disorders

The skin has a high turnover rate and can be removed without complicated surgical procedures if required; hence, it represents a good model for monitoring of treatment. Applications for cutaneous delivery of therapeutic siRNA are emerging owing to a strong demand for effective treatments of various skin disorders. Although successful studies have been performed using several different delivery techniques, most of these techniques encounter limitations for translation into the clinic with regards to patient compliance.

Pachyonychia congenita (PC) is an extremely rare, highly disabling autosomal dominant inherited disorder with less than a few thousand cases worldwide (Kaspar, 2005). This disorder which affects nails, skin, oral mucosa, hair and teeth (Smith et al., 2005), causes painful plantar calluses for which no satisfactory treatment is currently available. PC is caused by mutations in either keratin K6, K16 or K17, including small deletions and single nucleotide changes. Selective depletion of the mutated keratin with siRNAs has the potential to directly target the molecular aetiology of the disease (Leachman et al., 2010). The lack of effective PC therapies, the accessibility of skin disease lesions and the ability to visually observe changes during treatment make PC a good human skin disease model for testing siRNA in a proof-of-concept trial for genetic disorders. TransDerm Inc. has developed an siRNA-based therapy for PC. Their compound, TD101 has been shown to specifically target the cytosine-to adenine single nucleotide K6a mutation (Leachman et al., 2008) and to reverse the mutant phenotype of cells in a dominant-negative tissue culture model by restoring their ability to form a structurally intact keratin intermediate filament network (Leachman, et al., 2010).

5.5 Cardiovascular pathologies

During the past few years many conceptual and technical advances have been made towards the therapeutic modulation of cardiac gene expression, leading to the identification of new therapeutically relevant targets.

One of the most important risk factors for cardiovascular disorders is hypercholesterolemia. RNAi approaches are under investigation for this disorder. Hepatic ABCA1 contributes to HDL plasma levels and influences lipemia. An adenovirus-mediated RNAi approach has been used to test the efficiency of plasmid-based siRNA-induced knockdown of cotransfected murine ATP binding cassette transporter A1 (ABCA1) (Ragozin et al., 2005). Compared to controls, Ad-anti-ABCA1 infected mice showed 50% reduction of endogenous ABCA1 and a clear upregulation of apolipoprotein E (ApoE). Similarly, in 2004 Alnylam scientists demonstrated that chemically modified siRNAs can silence an endogenous gene encoding apolipoprotein B (ApoB) after intravenous injection in mice resulting in reduction of total cholesterol (Soutschek, et al., 2004). These siRNAs were also shown to silence human

ApoB in a transgenic mouse model. Additionaly, ApoB specific siRNAs encapsulated in SNALPs, and administered intravenously, were shown to silence ApoB in non-human primates, and resulted in dose-dependent silencing of ApoB mRNA in the liver, with >65% lowering of cholesterol and >85% lowering of LDL. Significant reductions in ApoB protein, serum cholesterol and low-density lipoprotein levels were observed as early as 24 h after treatment and lasted for 11 days at the highest siRNA dose, thus demonstrating an immediate, potent and lasting biological effect of siRNA treatment. These findings show clinically relevant RNAi-mediated gene silencing in non-human primates (Zimmermann et al., 2006). Similarly intravenous administration of an ApoB-specific siRNA modified with a dendritic poly-L-lysine (KG6) resulted in knock-down of ApoB in healthy C57BL/6 mice without hepatotoxicity, and a significant reduction of serum lipoprotein cholesterol in apoliporpotein E-deficient mice (Watanabe et al., 2009). In 2006, Alnylam scientists presented data on a new target for the treatment of hypercholesterolemia. They showed that silencing the proprotein convertase subtilisn/kexin type 9 (PCSK9) a protein involved in the regulation of LDL cholesterol in mice resulted in meaningful reductions in cholesterol levels, yielding the first *in vivo* evidence that pharmacologic targeting of PCSK9 can result in potential therapeutic benefit (Duff & Hooper, 2011).

5.6 Ophthalmology

Perhaps the greatest success of siRNA technology has come from local administration of siRNAs to the eye. The eye is a relatively isolated compartment with low content in RNAses and limited access to the immune system. This relative isolation of the eye allows for a reduced systemic exposure to the drug and thus a reduction in possible toxic side effects. Several eye diseases have been approached with siRNA-based therapeutics, including wet age-related macular degeneration (AMD) and diabetic retinopathy (DR). These diseases are the two main causes of irreversible vision loss in the developed world. in fact, the first approaches to target human diseases with siRNAs aimed at the treatment of AMD and, currently, the eye is the focus of several R&D programs (see section 8 for more information on this topic).

Dry eye syndrome is a prevalent disease that affects visual acuity, activities of daily living and quality of life. Symptoms accompanying dry eye syndromes include itching, burning and irritation of the ocular tissues. Its severity can range from very mild disease to extremely severe cases with vision-threatening consequences. A more severe form of dry eye occurs in patients with Sjogren's syndrome. Further taking advantage of the ocular environment, Sylentis is developing an anti-TRPV1 siRNA compound for the treatment of ocular discomfort associated to dry eye syndrome.

Retinitis pigmentosa (RP) is a leading cause of inherited blindness. This disorder involves photoreceptor-cell degeneration and affects ~ 1 in 3000 people (Farrar et al., 2002). Mutated forms of the rhodopsin (RHO) produce a toxic protein in the retina that kills cells that receive light. Although great efforts have been made to explore new gene therapies for RP, inter and intragenic heterogeneity represents significant barriers to a therapeutic development. For example, more than 100 mutations in the human *RHO* gene, which encodes the photosensitive pigment in rod photoreceptors, have been identified in autosomal dominantly inherited RP (adRP) (O'Reilly et al., 2007). Development of therapies for each individual mutation would be technically difficult to achieve and not economically viable; thus a therapeutic approach that circumvents mutational diversity would be of great value. Several approaches have been made with this aim but none of them have successfully passed beyond R&D so far.

5.7 Cancer

In general, there are three potential targets for cancer therapies: genes that are part of cancer–associated pathways, genes involved in tumor-host interaction or those that are part of chemo-or radiotherapy resistance (Gartel & Kandel, 2006; Grimm & Kay, 2007; Takeshita & Ochiya, 2006). The most promising oncogene targets are those associated with malignant transformation. These genes are usually amplified, mutated and amplified as a result of chromosome or gene rearrangement or exogenously introduced by transforming viruses in human tumours. Although multiple genetic mutations have been identified in most human tumours it is often not clear which of these mutant oncogenes are the actual cause of tumorogenesis (Brummelkamp et al., 2002b). Oncogenes found in human cancer often differ by only a single base mutation from their wild-type counterparts required for viability of normal cells. No treatments are available to date to specifically inactivate just the mutant version of such oncogenes. Hence, RNAi has become a logical strategy to selectively target each of these mutant alleles that define oncogenesis, including novel fusion proteins that define many types of cancer (Ameyar-Zazoua et al., 2005). There is emerging evidence that siRNA may represent a novel therapeutic modality for cancer treatment when optimized local and systemic delivery systems are available. Researchers at the Netherlands Cancer Institute reported successfull retroviral delivery of siRNAs that leave the wild-type K-RAS allele untouched while specifically inhibiting the mutant K-RASV12 allele in human pancreatic carcinoma, leading to loss of tumorigenicity and persistent loss of oncogenic phenotype (Brummelkamp, et al., 2002b). This is the first evidence in living animals that cancer can be controlled by blocking the expression of a single mutant protein using RNAi. HPV plays an important role in causing cervical cancer. Specific knockdown of HPV18 E6 and E7 oncogenes has been achieved resulting in efficient inhibition of the growth of HPV-positive cervical cancer cells (Wise-Draper et al., 2005). Different lentiviral RNAi approaches have been used to target different oncogenes. For example, researchers introduced a lentiviral vector into glioma cells targeting bcl-2, whose overexpression mediates protection from apoptosis. Delivered alone or in combination with another vector expressing a secreted form of TRAIL, a TNF family member, a significant reduction of tumours was achieved after their transplantation into nude mice (Kock et al., 2007). Similarly, others used a lentiviral RNAi to inhibit BRAF and Skp-2 genes, that are frequently up-regulated and mutated in melanoma cells, showing significant anti-tumour efficacy (Sumimoto et al., 2005). Fusion oncogenes, especially prevalent in lymphoproliferative cancers are attractive targets for RNAi, as chimeric mRNA is unique to tumour cells. The bcr-abl fusion oncogene in chronic myelogenous leukemias resulting from translocations t(9;22) is an example. Sengupta and collaborators targeted the bcr-abl junction in primary CD34+ CML cells using an Epstein-Barr virus-encoded shRNA in primary CD34+ CML cells which led to apoptosis (Sengupta et al., 2006). RNAi in combination with anticancer drugs has also been investigated in mice to test whether a combination therapy can promote apoptosis and reduce tumour burden. Small molecule inhibitors, such as Imatinib, are effective therapies for tyrosine kinase fusions BCR-ABL-TEL-PDGFβR-mediated human leukemias, but resistances are often developed. The unique fusion junctions of these molecules are attractive candidates for RNAi. Chen and co-workers have developed a retroviral system for stable expression of siRNA directed to the unique fusion junction sequence of TEL-PDGFβR oncogene (leads to leukemia in humans) in transformed hematopoietic cells. Stable expression of the siRNA resulted in approximately 90% inhibition of TEL-PDGFβR expression and its downstream effectors. Inhibition of this gene extended disease latency and survival of mice that received

transplants, but also mediated synergism with Imatinib (Chen et al., 2004). Similarly Landen and collaborators demonstrated that inhibition of EphA2 by siRNA resulted in a 50% of reduction of tumor size and its combination with paclitaxel resulted in a 50% reduction of tumor size in ovarian cancer cells (Landen et al., 2005). RNAi strategy has also been described to effectively silence other cancer related genes such as mutant forms of P53, KRas (Berns, 2010) to increase chemosensitivity by RNAi, silencing thymidylate synthase (TS) mRNA (Schmitz et al., 2004) or drug sensitivity and resistance genes (McCarroll et al., 2010), or to induce cancer immunity by the RNAi-induction of potent antigenic determinants and the immune-mediated rejection (Pastor et al., 2010).

6. Targeting microRNAs: A new paradigm and emerging field in drug development

6.1 Mechanism of action of miRNAs

MicroRNAs are small non-coding RNA molecules of approximately 22 nucleotides that regulate gene expression of their target genes usually at the post-transcriptional level (Bartel, 2004). miRNAs are synthesized as pri-miRNAs by polymerase II (Figure 1). Pri-miRNAs are processed in the nucleus by an RNase III enzyme named Drosha to generate the so called pre-miRNAs (Lee et al., 2003). Pre-miRNAs are transported from the cell nucleus to the cytoplasm by exportin-5 where the final cleavage takes place (Lund et al., 2004). The final cleavage is performed by another RNase III enzyme called Dicer, which yields the mature functional miRNAs. The mature double-stranded miRNAs are dissociated and one of the strands is loaded into AGO proteins resulting in the assembly of the miRNA ribonucleoprotein complex (miRNP). This strand then guides the miRNP complex to the target. In contrast to siRNAs, miRNAs are not in general perfectly complementary to their target mRNA. miRNA interact with their target mRNA by small "seed sites"; which are base pairings between 2 and 8 nucleotides of the miRNA with the 3′ UTR region of the target mRNA. Binding of the miRNP to its target leads to repression of the target gene by translational inhibition of the target mRNA (Wightman, et al., 1993). Depending on the degree of miRNA-mRNA complementarity, miRNA can induce an AGO-mediated degradation of the target mRNA, thus regulating gene expression by reducing mRNA levels (Hutvagner et al., 2001). Although miRNAs usually silence gene expression, recent work has shown that a few miRNAs are able to enhance the expression of their target genes (Orom et al., 2008). Enhancement of gene expression is achieved by using miRNA binding sites located in the 5′ UTR of the target mRNA.

The seed region of a miRNA is of particular interest in targeting and is also the most evolutionary conserved region of miRNAs. According to the 17th version of the miRBase released in April 2011, 1424 miRNAs have been identified in humans and 720 in mice. Because miRNAs do not require being perfectly complementary to their target sequence one microRNA is potentially able to affect hundreds or even thousands of targets. This mechanism of regulation indicates that miRNAs could regulate a given phenotype by regulating the expression of a single upstream key target or by regulating several targets in the same biological route. It should be mentioned however that miRNAs do not usually robustly modulate a single mRNA, but it is its ability to affect multiple targets that allows them to be key regulators of most biological processes. The activity of miRNAs acts as an amplification mechanism in which the actual number of identified miRNAs could potentially regulate most of the human genes. Given the prominent role miRNAs have on

gene expression it is not surprising that alterations in their expression profile can lead to disease and to alterations in the mechanisms of drug action (Rukov & Shomron, 2011). As a consequence, multiple pharmaceutical and biotechnology companies have focused their efforts on miRNAs as therapeutic targets and diagnostic biomarkers.

6.2 Role of miRNAs in disease

The ability of miRNAs to regulate gene expression at a very upstream level point out that dysregulation of miRNAs can lead to multiple pathologies. Concomitantly, the machinery that leads to the biogenesis of miRNAs has been profoundly studied, especially at the level of Dicer, and its alteration has been shown to have devastating consequences. Loss of Dicer1 leads to lethality early in development, with Dicer1-null embryos depleted of stem cells (Bernstein et al., 2003). Because Dicer1 knock-out mice are lethal, Dicer1 has been selectively knocked-out in most tissues. The consequences of deleting Dicer1 are multiple and reflect the key role of miRNAs in gene regulation. Targeted deletion of Dicer1 from specific tissues or cells has been shown to alter cell cycle regulation and enhance tumor susceptibility (Sekine et al., 2009), dysregulate immune cell differentiation (Koralov et al., 2008; Muljo et al., 2005), cause autoimmunity (Zhou et al., 2008), impair neural development (Davis et al., 2008; Huang et al., 2010), alter central nervous system function and promote neurodegeneration (Damiani et al., 2008; Hebert et al., 2010), impair pancreatic islet cell genesis (Lynn et al., 2007), cause dilated cardiomyopathy and heart failure (Chen, 2008), impair spermatogenesis (Hayashi et al., 2008), dysregulate skin development (Yi et al., 2009), prematurely stop inner ear development (Soukup et al., 2009), alter smooth muscle function (Albinsson et al., 2010) and impair prostate development (Zhang et al., 2010).

One of the most critical observations in miRNA pathobiology is the recognition that miRNAs modulate the transcriptome to promote or to attenuate cancer predisposition. miRNAs have a critical role in regulation of cellular differentiation, angiogenesis, survival and growth. Additionally, global repression of miRNA seems to be a common feature in most cancers, including lung cancer, breast cancer, colon cancer, pancreatic cancer and leukemia (Croce, 2009). Remarkable changes have been observed in the miRNA expression pattern between tumours and normal tissue (Volinia et al., 2006; Yanaihara et al., 2006) and approximately 10% of all the up to date described miRNA have been shown to be altered in pulmonary fibrosis (Pandit et al., 2011).

Myocardial miRNA profiling has led to the identification of more than 200 miRNAs that are consistently expressed in heart tissue. Many of these miRNAs are deregulated in heart disease. Several miRNAs that control the switch between foetal and adult enzymes have been identified. Deregulation of these miRNAs has been linked to cardiomyocyte hypertrophy, ventricular septal defect and heart failure (van Rooij et al., 2009).

During the past several years miRNAs have emerged as key regulators of immune cell lineage commitment, differentiation and maintenance of both innate and adaptive immune response (Baltimore et al., 2008). Dysregulation of the checkpoints that maintain immune tolerance is one of the hallmarks of autoimmunity. Regulatory T cells (Tregs) suppress the activation of effector T cells to maintain immune system homeostasis and tolerance to self-antigens. Ablation of miR-14 in Treg cells and overexpression of miR17-92 in lymphocytes have shown to promote autoimmunity in mouse experimental models (Kohlhaas et al., 2009). Additionally, deregulation of the normal pattern of miRNA expression has been identified in patients of systemic lupus erithematosus, rheumatoid arthritis and multiple

sclerosis (Dai & Ahmed, 2011). In addition to autoimmunity, miRNAs have been found to have a role in other alterations in immunity such as psoriasis, a chronic inflammatory disease.

Several miRNAs have been found to be differentially expressed in the central nervous system (CNS) (Fiore et al., 2008). These CNS-restricted miRNAs have a role in neuronal development and function. As seen in other organs, deregulation of the normal pattern of expression of these miRNAs can lead to disease, in the case of the central nervous system, to neurodegenerative diseases. Although miRNA expression profiling has been achieved with different degrees of success for some neurodegenerative diseases such as AD, PD and HD, it should be mentioned that miRNA profiling is particularly challenging in the central nervous system. Firstly, some of the miRNAs are only expressed in very small populations of neurons or non-neuron cells in specific brain areas; secondly due to the particularities of neurodegenerative diseases, it is difficult to obtain healthy and diseased tissue from the same patient. This latter reason has raised issues when addressing the role of miRNAs in initiating the disease rather than being a secondary factor caused by deregulation of biological pathways. Conditional inactivation of Dicer in neurons has shed some light to this issue. Selective deletion of Dicer in dopaminergic neurons in mice leads to ataxia, front and hind limb clasping, reduced brain size and smaller neurons (Cuellar et al., 2008). Deletion of Dicer in other brain regions such as the hippocampus and in Purkinje cells in the cerebellum cause alterations that indicate that dysregulation of miRNA expression could be contributing to neurodegeneration (Schaefer et al., 2007), however effort should be made in the near future in elucidating the role of individual miRNAs in neurodegeneration in order to identify potential biomarkers and targets for the development of new drugs.

As summarized above, miRNAs have a crucial role in development of diseases. Dysregulation of gene expression is a common feature of most pathologies, thus it shouldn't be surprising that in the coming years alteration in the miRNA profiles are found for virtually every disease. Addressing the impact of these alterations on pathobiology is essential in order to develop clinically useful miRNA based therapies.

Some examples of the alteration of miRNAs in disease are shown in Table 1.

6.3 miRNAs as diagnostic biomarkers

Because of the crucial role of miRNAs in a plethora of diseases considerable effort has been made to identify specific miRNA profiles that could serve as biomarkers to help in the diagnosis of particular diseases or indicate the eventual course of a given disease. Some promising data has been obtained in the diagnosis of cancer where Rosenfeld and coworkers successfully profiled over 130 metastases of unknown tissue origin and were able to identify the tissue origin of the primary tumour with a 90% accuracy studying the miRNA profile (Rosenfeld et al., 2008). miRNA profiling has also proven useful in predicting the survival rate of cancer patients. Poor survival rate has been linked to high levels of miR-21 in colon adenocarcinoma (Schetter et al., 2008) and a combination of low levels of let-7a and high levels of miR-055 correlate with poor prognosis in lung adenocarcinoma (Yanaihara, et al., 2006). Several studies have also addressed the potential role of circulating miRNA profiling. miRNAs are stable in serum and plasma and their expression in blood is reproducible and, in some cases, indicative of the diseased state (Mitchell et al., 2008). miRNA serve as diagnostic markers in plasma and serum isolated from human patients with distinct forms of cancer (Kosaka et al., 2010). Correlation between levels of certain miRNAs and cancer has been found for lung cancer, hepatocellular carcinoma, pancreatic cancer, gastric cancer and

CLL among others (Ceman & Saugstad, 2011). The sensitivity of some of these methods has shown to be greater than traditional and more invasive diagnostic methods (Xie et al., 2009). Specific patterns of miRNA expression have been identified in plasma samples of rats following tissue injury (Laterza et al., 2009). Plasma levels of miR124 have arisen as a promising candidate biomarker for cerebral infarction (Wang et al., 2011) and correlation has been found between the plasma levels of miR17 and miR20 and multiple sclerosis (Cox et al., 2010). Additionally, modifications of the pattern of expression of miRNAs could eventually anticipate how a certain individual would respond to a particular treatment. This kind of approach has yielded some success in cancer, where miR-21 has been identified as a biomarker for early detection on non-small cell lung cancer and its levels are related to the sensitivity to platinum-base chemotherapy (Wei et al., 2011). It should be noted that, if successful, this approach is especially attractive due to the low invasiveness of the technique.

miRNA	**Disease**	**Change in disease**
miR-15a/16-1, miR-29b, miR-181b, miR-34	Chronic lymphocytic leukemia	Downregulated
miR-21, miR-155	Pancreatic cancer	Upregulated
let-7, miR-1, miR-29, miR126	Lung cancer	Downregulated
miR-17-92, miR-21, miR-155, miR-221/222	Lung cancer	Upregulated
let-7, miR-29, miR-30	Idiopathic pulmonary fibrosis	Downregulated
miR-126, miR-210	Ischemic myocardium infarct	Upregulated
miR-203, miR-146	Psoriasis	Upregulated in skin (miR-203), upregulated in immune cells (miR-146)
miR-125a	Lupus erithematosus	Downregulated in T cells
miR-148a , miR-21	Lupus erithematosus	Upregulated in CD4+ T cells
miR-146a	Rheumatoid arthritis	Upregulated in selected cells
miR-124[a]	Rheumatoid arthritis	Downregulated in synoviocytes
miR-326	Multiple sclerosis	Upregulated in CD4 T cells
mi-R34a, miR-155 , miR-326	Multiple sclerosis	Upregulated in MS lesion
miR-9, miR-25b, miR-128	Alzheimer's disease	Upregulated in hippocampus
miR-124[a]	Alzheimer's disease	Downregulated in hippocampus
miR-133b	Parkinson's desease	Downregulated in midbrain

Table 1. Some examples of miRNAs dysregulated in disease

6.4 miRNA therapeutics

Deregulation of miRNAs significantly modulates biological pathways. Alteration in these pathways cannot only lead to disease but also alter the way an organism responds to a drug.

miRNA expression profile can successfully be manipulated in order to modulate genes that cause disease or to alter the natural response to a given drug. For diseases in which a reduction in certain miRNAs underlies the diseased condition, re-introduction of the miRNA could be of therapeutic benefit (miRNA mimics). For those diseases in which overexpression of miRNAs are found, inhibition of the miRNA function could restore the proper pattern of gene expression (miRNA inhibitors).

6.4.1 Target validation

One of the first challenges in miRNA therapeutics is identification and validation of miRNA targets. Validation strategies usually imply proving the interaction between miRNAs and the predicted mRNA targets, demonstration of co-expression of the miRNAs and the target mRNAs in the same cell population, proving a predictable effect of the miRNA on target protein expression and finally demonstrating that miRNA mediated regulation of its target mRNAs leads to changes in biological function (Kuhn et al., 2008). In silico analysis largely facilitates the prediction of possible miRNA-mRNA interactions, thus predicting potential targets. Multiple algorithms have been developed in order to help predict the interaction between miRNAs and their targets (Lewis et al., 2003; Rajewsky, 2006). These analysis are generally based on complementary analysis between the seed region of the miRNA and the 5'UTR of the potential target mRNA. However, in order to demonstrate that a given mRNA is indeed target of a certain miRNA, reporter assays have to be carried out (Nicolas, 2011). To further validate miRNA targets *in vitro* experiments are usually performed in which mRNA profiles of cells or tissues either overexpressing or lacking a given miRNA are compared with wild-type cells or tissues. An alternative approach to demonstrate miRNA-mRNA interaction is to take advantage of the miRNA-mRNA-AGO interaction to form the miRNP complexes. miRNP complexes can be immunoprecipitated followed by generation of cDNA libraries which can then be analysed by microarrays (Beitzinger et al., 2007). Finally, gain or loss of function *in vivo* studies are needed to assess the biological function of miRNAs in health and disease (Kuhn, et al., 2008).

6.4.2 Therapeutic options, chemistry and delivery

Several approaches can be used when aiming to restore the normal function of miRNA mediated gene modulation. Increasing the function of a given miRNA can be achieved by re-introducing the miRNAs in the target cell population or by reactivation of its biogenesis by manipulating gene expression using a conventional small molecule strategy. Reduction or even inhibiting the action of miRNAs can be approached by inhibiting its expression using either conventional strategies, that target transcription factors, which would in turn decrease its gene expression, or innovative RNAi based technologies. Introduction of miRNA mimics or other types of oligonucleotides in order to decrease the expression of miRNAs using the cellular RNAi machinery requires delivering stable oligonucleotides into the target cells or tissues. Biological cell membranes are natural barriers for oligonucleotides, which are high molecular weight, highly charged polyanionic molecules. By introducing chemical modifications in the oligonucleotides, some of the hurdles these molecules face when trying to reach their target organs have been addressed. Some of the nucleotide modifications developed in order to increase stability of the molecules have collaterally increased their delivery rate as well. Some of these modifications include 2'-O-methyl modification of RNA, phosphorothioate linkage, modifications in the 2' ribose and LNAs.

Oligonucleotides carrying these modifications have been found to be effective in both cell cultures and mice (Seto el al., 2010). The success of these modifications has allowed Santaris Pharma to develop one compound, carrying LNA modifications in its structure, to phase II clinical trials. Oligonucleotide analogs are also being used hoping to improve stability of miRNA inhibitors and to increase the affinity of the miRNA-mRNA target interaction; these analogs include peptide nucleic acids (PNA) and morpholinos. Although morpholinos have demonstrated their efficacy when injected in zebrafish embryos (Moulton & Jiang, 2009) and PNA oligonucleotides have been used to inhibit the expression of miRNAs in cell culture (Oh et al., 2011), *in vivo* validation in small animal models has yet to be carried out.

Depending on the therapeutic goal and the target tissue viral vectors may be used to introduce miRNAs into cells. The obvious advantages of using viral vectors for delivery of miRNAs are efficient delivery to the target organs and increased over time production of the transgene. Retroviruses, adenoviral vectors, AVV and lentiviral vectors have been widely used to express miRNA mimics in cells (for a recent review see (Liu & Berkhout, 2011)). Viral vectors have also been developed to inhibit miRNA function, these vectors express miRNA target sites to inhibit specific miRNAs or a miRNA family from regulating their natural targets, they are the so called "antagomirs". These novel approaches have been used to target multiple miRNAs and have already demonstrated efficacy in mouse models (Krutzfeldt et al., 2005).

miRNA-targeted therapies can be systemically or locally administered, and recent efforts have been aimed towards targeted delivery of drugs. Systemic delivery of oligonucleotides is challenging due to the poor biodistribution achieved, different strategies have been used to improve this drawback. Recently polyethilenimine (PEI)-mediated delivery of miRNA has shown promising results in mouse models of cancer (Ibrahim et al., 2011), in this study miR-145/PEI complexes were administered systemically resulting in unmodified miRNA molecules being delivered into the tumors where the miRNA was shown to exert its biological activity. Encapsulation of the nucleic acid with cationic lipids or polymers is an attractive field of research. Such an approach has recently been validated *in vivo* for the delivery of miR-122 in liver where it has been shown to modify gene expression resulting in lowering plasma cholesterol levels (Su et al., 2011). Some of the oligonucleotide modifications developed to improve stability of the nucleic acids have also improved the delivery of miRNAs to tissues where systemic delivery is most effective such as liver, jejunum, lung and liver (Seto, 2011). Some of these modifications include cholesterol-conjugation of miRNAs and LNA-modified oligonucleotides.

Local delivery is very attractive in organs that are easily accessible such as the eye or the lungs. Local delivery ensures delivery to the correct organ and diminishes the eventual off-targets but can only be used in cases where accessibility is not an issue. In a recent publication McArthur and colleagues demonstrate that intravitreal administration of miR-200b prevented diabetes-induced increased vascular endothelial growth factor (VEGF) mRNA and protein in a rat model of diabetic neuropathy (McArthur et al., 2011).

Targeted delivery of miRNAs implies physically steering the microRNA molecule to a given tissue. Targeted delivery can be achieved by using viral vectors with specific tropism for a tissue. Some serotypes of AVV virus show natural tropism for the heart. These viral vectors have been reengineered with therapeutically desirable properties while maintaining the tropism for the heart. These vectors combined with cardiac tissue specific promoters have been used to transfer RNA-based therapies with exceptional outcomes and are already

undergoing translational clinical trials (Poller et al., 2010). Further approaches used for target delivery of siRNAs can be applied to miRNAs.

6.4.3 Specificity and off targets

miRNA based therapies are potentially very powerful since targeting miRNAs can affect a biological pathway at different levels regulating multiple gene targets simultaneously. At the same time, precisely the fact that makes miRNAs promising therapeutic targets is the source of their major drawback for these kind of approaches: the unanticipated OTEs leading to safety issues. It should be noted that when introducing miRNA mimics sequence optimization and off targets are not usually big concerns since miRNAs have naturally evolved for optimal targeting. However when high doses of the miRNA mimics are administered or when miRNA mimics are introduced into cell populations or tissues that do not naturally express the miRNA, unforeseen toxic effects of these molecules could arise and should be taken into consideration when assessing these strategies (Seto, 2011).

6.5 Current status of miRNA-targeted compounds

In May 2008, Santaris Pharma, was the first company to receive approval to start clinical trials with a miRNA targeting drug. The compound, named miravirsen, targets miR-122, a liver specific miRNA involved in hepatitis C replication. Currently miravirsen is undergoing Phase II trials and is the most advanced miRNA targeted compound.

miRagen is a pharmaceutical company currently developing products targeting miRNAs. Four compounds with two different indications in their pipeline are currently in pre-clinical stages. The first two target miR-208 and miR-499 respectively, and are for chronic heart failure whereas the other two target miR-15 and miR-195 for the treatment of post-myocardial infarction remodelling. Other compounds in miRagen pipeline are focused on cardiovascular disease and ALS.

Regulus is advancing miRNA therapeutics in several areas. Its fibrosis program is developing compounds that target miR-21, pre-clinical studies have demonstrated a role for this miRNA in different fibrosis models including heart, lung, kidney and liver, miR-21 is also under study in the company's oncology program. Other miRNAs being targeted include miR-122 for hepatitis C, miR-155 for anti-inflammatory diseases and miR-33a and b for metabolic diseases.

Mirna Therapeutics Inc. is also developing eight lead candidates as part of their oncology program. Some of the miRNAs targeted are let-7, miR-34 and miR-16.

Rosetta Genomics main focus is on developing diagnostic tests for cancer. Several diagnostic kits are currently in their pipeline, e.g., miRview bladder will be able to predict the risk of superficial bladder cancer to become invasive and miRview lung is aimed towards the differentiation of neuroendocrine tumors from non small cell lung cancer tumors using small preoperative biopsies. In the field of therapeutics, Rosetta genomic's is putting together efforts with Regulus in miRNA targeted therapeutics targeting miRNAs that have a potential role in hepatocellular carcinoma.

6.6 Future prospects of miRNA targeted therapeutics

Targeting human disease with miRNA-targeted therapies represents a very powerful therapeutic approach. Enormous advances in understanding the biology of miRNAs have

been made in the past decade. These lessons are a fundamental basis to approach miRNA targeting with rational design, focused on enhancing target affinity, stability and tissue uptake. Despite the huge therapeutic potential of miRNA regulation, this novel therapy still faces some major challenges; addressing OTEs is at the top of the list. However, the impact of off targets will be greatly reduced with improved mechanisms of delivery. In this regard, targeted delivery represents some very interesting features including local delivery to the target sites combined with reduced systemic exposure. Improvements in *in silico* programming are also needed in order to increase the accuracy of miRNA target identification. With one compound already in clinical trials, the future of miRNA-targeted therapies depends on our ability to translate the accumulated knowledge on miRNA biology into the clinic.

7. Advantages of RNAi-based therapeutics and challenges ahead

RNAi technology presents many advantages for therapeutic development with respect to classical compounds, which are centred on the following points:

- They are based on an endogenous mechanism and involve the administration of a type of molecule already present in the cells. Hence, in principle, they may be less hazardous: cells should have the capacity to handle resulting breakdown products.
- They can potentially address any pathological target, which means that even diverse intracellular molecules could be addressed.
- RNAi-based therapeutics are considerably more potent than antisense molecules before them (Bertrand et al., 2002) and, consequently, may be used at lower concentrations. They are believed to have a more persistent pharmacological action than traditional drugs because they block protein synthesis and, hence, the cell will have to re-synthesise the protein from scratch to return to its previous state. Thus allowing the use of lower or less frequent doses.
- Given the high selectivity and specificity of these compounds, they may potentially have less harmful side effects.
- Another main advantage from an industrial perspective is their much shorter pharmacological development, ~2-3 years from proof of concept vs ~4-6 years for traditional compounds. This can be attributed, among other things, to the fact that the compounds can be designed against homologous regions between the human gene sequence and the different animal models used for preclinical studies, thus simplifying toxicology studies.
- Finally, despite being novel entities, they are easy to manufacture using a nucleotide synthesiser, which simplifies large scale production. Since these compounds are chemical entities rather than biological products, the regulatory process for approval of these medicaments is greatly simplifyed.

Nevertheless, RNAi still has certain hurdles to overcome before its full potential can be exploited. The main obstacle is delivery to the desired tissues. Although many advances have been made in this area, much work still remains for the complete therapeutic possibilities to be fully unveiled. On the other hand, in a clinical setting, RNAi can only be used to treat pathologies caused by expression or overexpression of a given protein or by the presence of exogenous organisms, as its mechanism works through suppression of protein expression, i.e., it is only of use when the therapeutic option requires a loss of function. Furthermore, although any gene is a potential target for RNAi, in practice not all

mRNAs are as easily silenced and new more sophisticated algorithms will need to be developed to overcome this issue. Last, but not least, the issue of OTEs resulting from siRNAs silencing unwanted genes can lead to important safety considerations when developing new medicaments.

8. RNA interference in the clinic: Current state and prospects for siRNA-based strategies

Considering RNAi was discovered just over a decade ago, this technology has advanced towards clinical trials with amazing speed. Initial most advanced compounds were designed to treat wet AMD taking advantage of the relatively RNAse free environment of the interior of the eye.

This disease causes loss of vision due to abnormal blood vessel growth (choroidal neovascularisation) behind the retina and macula. Bleeding or leaking of fluids from these newly formed blood vessels causes the macula to bulge or lift, resulting in irreversible damage and loss of vision if left untreated. Anti-VEGF agents cause regression of abnormal blood vessel growth and improvement of vision. In 2004 the FDA approved Pegaptanib (Macugen®, OSI Pharmaceuticals, Pfizer), a VEGF targeting RNA-aptamer to be administered by intravitreal injection and which was the first antiangiogenic therapy to be approved. Later, Lucentis was approved, a monoclonal antibody also against VEGF, which is again administered through intravitreal injection and which is more effective at maintaining and restoring vision.

Based on RNAi, Bevasiranib (a VEGF siRNA developed by Acuity Pharmaceuticals and later by Opko Health) and Sirna-027 or later AGN211745, developed initially by Sirna Therapeutics and licensed to Allergan, targeting VEGF-receptor, were developed with the same therapeutic objective (AMD). Both advanced through phase II clinical trials and Bevasiranib initiated a Phase III study, having shown very promising results in animal models. However, in 2009 both compounds were halted within a few months of each other, due to not improving efficacy over Lucentis, the reference treatment. Also, Quark Pharmaceuticals developed an siRNA for the treatment of AMD targeting the hypoxia-inducible RTP801 gene involved in disease progression, which was later licensed to Pfizer. This compound has reached Phase II, and Quark announced recently their intention to initiate a Phase 2b study to determine the correct dose necessary to produce a therapeutic effect sufficiently superior to the current standard of care to benefit patients over the long term given new emerging therapeutic modalities.

Quark Pharmaceuticals has another two compounds undergoing clinical development. Firstly QPI-1002, an siRNA which targets p53, thus inhibiting apoptosis, is under development for the treatment of acute kidney injury and also for delayed graft function following renal transplantation. Both indications are currently in phase II clinical trials and in August 2010 Quark granted Novartis an option for a worldwide exclusive license for all indications of this compound. Furthermore, QPI-1007, an anti-caspase 2 siRNA, has recently initiated a phase I trial for the treatment of non-arteritic anterior ischemic optic neuropathy. This compound is expected to inhibit apoptosis of retinal ganglion cells thus preventing vision loss ensuing in these patients. QPI-1007 is also under earlier stages of development for the treatment of glaucoma, which also leads to blindness through damage of the optic nerve, in this case as a consequence of elevated intraocular pressure.

Also taking advantage of the peculiarities of the eye's environment, Sylentis has developed a compound, SYL040012, for the treatment of ocular hypertension and glaucoma presently undergoing a Phase Ib clinical trial. This compound inhibits expression of adrenergic receptor β2 in tissues of the ocular anterior chamber, resulting in a decrease of aqueous humour production and consequent reduction of the pressure inside the eye. Just one step behind in its product pipeline is SYL1001 for the treatment of ocular pain associated to dry eye syndrome, poised to enter the clinic this year.

Another of the most advanced siRNA compounds in the clinic, again in Phase II, is Alnylam Pharmaceutical's compound for the treatment of RSV infection. This compound, ALN-RSV01, targets the nucleocapside encoding gene of the virus and therefore inhibits viral replication in the lung. This compound is administered intranasally directly to the lung and is partnered with Kyowa Hakko Kirin in Asia, and with Cubist Pharmaceuticals for the rest of the world.

Further programs in the clinic developed by Alnylam include their compound ALN-VSP, for the treatment of liver cancers and potentially other solid tumors, currently in Phase I. This RNAi therapeutic targets two key genes each involved in the disease pathway of liver cancer: kinesin spindle protein, or KSP, involved in cancer proliferation, and vascular endothelial growth factor, VEGF, involved in the growth of new blood vessels that feed tumors. Also, as part of their new 5x15 programs, ALN-TTR, an RNAi therapeutic targeting transthyretin (TTR) for the treatment of TTR amyloidosis (an orphan hereditary disease which results in toxic protein deposits in several tissues) entered Phase I trials in early July 2010.

As previously mentioned in section 5.4, TransDerm Inc. has developed an siRNA for Pachyonychia congenital disorder which underwent a phase I clinical trial. However, upon completion the company announced it was directing its resources at developing an enhanced delivery system that was less painful than the intradermal injections used in this trial.

A still unsolved problem in medicine is the treatment of HIV infection, and one of the pioneering RNAi compounds in clinical trials was termed pHIV7-shl-TAR-CCR5RZ, initially developed by Benitec Inc. This compound was in a Phase I/II pilot study from 2007 to 2010 in collaboration with City of Hope Research Hospital. It is based on expression of a construct producing specific shRNA specific for the above targets introduced into CD34+ blood progenitor cells. According to Benitec's website, this study is now being progressed by the research and clinical team at the City of Hope Research Hospital.

A further clinical setting with multiple participants is the treatment of cancer, with different compounds in Phase I clinical trials. This includes Atu027 developed by Silence Therapeutics for the treatment of advanced, recurrent or metastatic solid malignancies. This compound, which targets PKN3 (a protein kinase C-related molecule believed to be an effector mediating malignant cell growth), was one of the first to advance in the RNAi landscape. However, it has suffered numerous delays in its move into the clinic due to the difficulties associated to delivery of these compounds. The company then developed a proprietary lipid delivery technology called AtuPlex. This platform is based on lipid components that embed siRNA into multiple lipid bi-layer structures. The resulting nanoparticle structure consists of the siRNA combined with a cationic lipid, fusiogenic lipid and PEG. This system was used to formulate Atu027, which was then administered in the clinical trial and produced positive results according to the company's abstract at ASCO meeting this year.

Further RNAi for the treatment of cancer include CALAA-01 for the treatment of solid tumours developed by Calando Pharmaceuticals. CALAA-01 is a siRNA which targets RRM2 (M2 subunit of ribonucleotide reductase) and is formulated in cyclodextrin-based particles. CEQ508 has been designed for the treatment of familial adenomatous polyposis (FAP) by targeting beta-catenin using tkRNAi. This technology, developed by Cequent Pharmaceuticals (now Marina Biotech), uses modified *E. coli* to deliver RNAi compounds to the intestine.

Another indication of interest to the pharmaceutical industry is the treatment of hypercholesterolaemia, with different companies having compounds under development. In clinical trials, Tekmira Pharmaceuticals has a compound TKM-ApoB (previously PRO-040.201) which targets expression of Apolipoprotein B that has completed a phase I trial.

The pioneer in the field of miRNA silencing is Santaris Pharma with its LNA technology. The most advanced compound is SPC3649, which targets miR-122, for the treatment of hepatitis C virus infection and currently in Phase II.

9. Navigating the patent thicket

At the turn of the century a revolutionary discovery was made in the field of molecular biology: the existence of RNA interference. Firstly in flatworms by original finders and Nobel laureates Andrew Fire and Craig Mello, and later its existence in mammalian cells by Tuschl and coworkers, opened the door to this truly limitless (or so it seemed) technology. Following the expected course of events characteristic of science in our era, the parties involved rapidly filed patent applications on their discovery. These initial filings were done by the academic institutions where discoveries were made, such as Carnegie Institution or Max Planck Institute; and were rapidly out-licensed to industry.

Fire and Mello's finding in flatworms generated a seminal patent—the Carnegie patent—which is available for licensing to any interested party and describes how double-stranded RNA silences target genes in a cell. The initial filing has given rise to a complicated patent family with many sister applications both in Europe and in the US, including different limitations such as length of the molecules, *in vitro* or therapeutic use, *in vivo* use in plants, in flatworms, etc.

Initial discoveries used molecules of 25 nucleotides or more in length, and at that size, the molecules can trigger dangerous immune responses, making them unsuitable for clinical use. However, it was later found that making the molecules smaller could avoid immune reactions while disabling mRNA. This discovery was made by 4 scientists—Thomas Tuschl at Max Planck Institute, Philip Sharp at MIT, Philip Zamore at UMass Medical School and David Bartel at the Whitehead Institute—and produced the fundamental patent known as Tuschl I, which covers both modified and unmodified siRNA molecules, 19 to 25 nucleotides long. In 2002, three of the four schools involved in the discovery agreed to license Tuschl I exclusively to Alnylam. UMass Medical School chose not to, and granted a nonexclusive license to CytRx (now RXi) for limited therapeutic rights. Then in 2003, Sirna took a coexclusive license to Tuschl I from UMass Medical School, the end result being that Sirna, Alnylam and CytRx (to a limited degree) can operate under Tuschl I. Further research on Tuschl's part then led to the finding that siRNA structures containing three prime overhangs were particularly advantageous. These results led to filing of the Tuschl II patent, which was licensed exclusively to Alnylam. Again, both of these patents have evolved into

complicated patent families with multiple sister applications both in Europe and the US covering specific features.

These filings led to an initial competition for dominance between two major companies: Alnylam Pharmaceuticals and Sirna Therapeutics. There was some controversy about the value of Tuschl I and Tuschl II over each other. While Alnylam assured the supremacy of Tuschl II, Sirna argued that Tuschl I contains most of the major features found in Tuschl II anyway. Both companies invested significant resources in building up their patent portfolio either through further licenses or internal filings on further chemical architecture and/or use of modified nucleotides in siRNA compounds. Consequently, most subsequent players in the field will have to negotiate licenses (or have already done so) with these companies for the development of their compounds, thus contributing to maintaining their dominant position.

In any case, all three of these families there have been some granted patents, some of these undergoing complicated opposition procedures, and there are also still applications undergoing prosecution despite the fact they were filed in 1999 and 2000. This makes the outcome of what will eventually be covered somewhat uncertain.

Despite the many licensing deals reported by the press during this period, and the active patenting endeavours of all parties, there has been relatively little litigation of the patents involved. One notable case was made by two companies Nucleonics and Benitec, arch rivals working on expressed RNA interfering compounds to treat viral infections. The costs of their legal battle led to the dissolution of Nucleonics in mid 2008, and to Benitec withdrawing its international operations and reducing costs in its native Australia.

The next visit to the courts was made in mid 2009 by Alnylam Pharmaceuticals, who originally licensed the Tuschl I patent family from co-applicants Max-Planck Institute, University of Massachusetts, the Massachusetts Institute of Technology and the Whitehead Institute for Biomedical Research. Alnylam shares therapeutic rights to the Tuschl I estate with Sirna Therapeutics (now Merck) and to a limited degree with RXi. In this case Alnylam and Max-Planck were accusing the other co-applicants of misappropriating features relating to 3'-overhangs, which are object of the Tuschl II patent family, licensed exclusively to Alnylam. This case would have been of great interest to all players in the field, as the exact scope of each patent family has yet to be definitively determined. However, the involved parties reached an agreement outside the courts as recently as March 2011, in which among other terms, UMass Medical School gained the right to sublicense rights to the use of 3' overhangs to Merck.

The most recent legal action also involves Alnylam Pharmaceuticals, who is being sued by one time partner Tekmira over inventorship of certain delivery technologies. Tekmira, which owns technology relating to delivering siRNA compounds known as SNALPs resulting from its acquisition of Protiva Biotherapeutics, is a leading developer of lipid nanoparticle delivery technologies. Both companies started working together in 2006 and struck a first licensing deal in 2007 followed by a manufacturing supply deal in 2009. According to Tekmira's spokesman the delivery technology used for Alnylam's compound ALN-VSP for the treatment of liver cancer is the rightful property of Tekmira, and Alnylam has abused its collaborator status by disclosing the technology and methods to a third party and incorporating these features into its own patent filings. At present this process is yet at an early stage and the outcome difficult to predict.

As compounds near marketing approval and the economic stakes become higher, it is likely that the Courts will become busier with patent litigations from this field, and the patent landscape will probably become clearer for all involved.

As the race to join the field gained traction, big pharma, including Novartis, Roche, AstraZeneca or Merck, joined the playground with big deals signed from the end of 2006 to early 2008, the most notorious being the acquisition of Sirna Therapeutics by Merck for US$1.1 billion in October 2006.

With the deepening in knowledge of RNAi, so did the awareness of its limitations and hence the R&D to overcome these issues evolved. The main obstacles to widespread use of RNAi products are its vulnerability to degradation by RNAses ubiquitous in biological fluids, and the difficulty in targeting the compounds to the desired tissue or organ within the body. Consequently, there has been extensive patent filing on different chemical modifications useful to enhance resistance to degradation without losing silencing efficacy. Most companies in the field developed specific combinations of modifications. The main players in this area being Alnylam, through its access to the IP developed by Isis Pharmaceuticals for the use of antisense oligonucleotides, but also Silence Therapeutics, Quark Pharmaceuticals, and Santaris Pharma. The filing in this area has been so aggressive that it is probably difficult to develop a novel siRNA containing modifications which doesn't require a license to at least one, if not more, of the different patents.

Although the resistance to degradation has been overcome by technology, a huge hurdle remains in the delivery of compounds to the target tissue. This issue still remains as the main obstacle for this drug class to really flourish, and many of the more recent deals in industry have been made between purely chemical development RNAi firms and those who had developed delivery solutions. For example, Silence with Intradigm.

Bearing in mind the above described landscape, when starting on a new project from a patent perspective, one must analyse which of all these patent rights will be infringed by the commercialization of the novel compound. This includes analysis of the fundamental patents, and also those relating to nucleotide modifications and routes and vehicles for their delivery. But this is not all, for there have also been a great number of filings on specific gene targets. In fact, if one studies the dynamics of patent publications citing siRNA, the first publications appeared in 2003 and rapidly peaked to a maximum of over 400 publications in 2005, with well over 300 patent applications citing siRNA being published every year since then. Therefore every new project requires extensive analysis of the landscape to determine the best option to obtain freedom to operate in an increasingly competitive and complex area.

Regarding delivery, many approaches have been developed but none has been fully satisfying, and possibly as a consequence, the field's pace has slowed down considerably. Also, the two most advanced compounds in the clinic, both for the treatment of wet AMD were halted during 2009 before reaching or finalising phase III, their sponsors explaining the compounds weren't meeting the end-point of improved efficacy over Lucentis, the standard of treatment for this disease and a monoclonal antibody targeting the same molecule: VEGF. The exact reason remaining unknown, but all these factors probably having played their part together with the general economic crisis, in the last year or so big pharma have pulled out of many of the RNAi deals, and/or closed down their nucleotide operations. Examples of this include Pfizer having closed down its Oligo Therapeutics Unit early this year; Roche announced in November 2010 it was terminating its efforts in RNA interference, and Novartis refusing a further option to extend its collaboration with Alnylam. Consequently, at present there are a handful of companies dedicated exclusively to RNAi with programs in early clinical stages. For an overview of these companies and their programs in the clinic see Table 2.

Although the pace in the RNAi field has slowed down considerably, it remains to be seen how this area will develop and if the full potential of this technology can be harnessed. In such a case, we may witness the full revolution of drug development.

Company	**Drug**	**Disease**	**Target**	**Clinical Stage**
Opko Health	Bevasiranib	Wet AMD	VEGF	Phase III
Allergan	AGN-211745	Wet AMD	VEGF-R	Phase II
Quark Pharm.	RTP801	Wet AMD	RTP-801i	Phase II
	QPI-1002	Acute Kidney Injury / Delayed Graft Function	p53	Phase II
	QPI-1007	NAION	Caspase-2	Phase I
Sylentis	SYL040012	Glaucoma	b2-adrenergic receptor	Phase Ib
Alnylam Pharmaceuticals	ALN-RSV01	RSV	RSV nucleocapsid	Phase II
	ALN-VSP	Liver cancer	KSP + VEGF	Phase I
	ALN-TTR	TTR amyloidosis	TTR	Phase I
TransDerm Inc.	TD-101	Pachyonychia congenita	Keratin 6A N171K mutant	Phase I
Benitec / City of Hope	pHIV-shl-TAR-CCR5Z	AIDS	HIV *tat rev*	Phase I
Silence Therapeutics	Atu-027	Solid tumours	PKN3	Phase I
Calando Pharm.	CALAA-01	Solid tumours	RRM2	Phase I
Marina Biotech	CEQ508	FAP	β-catenin	Phase I
Gradalis Inc.	FANG	Solid tumours	Furin	Phase II
Duke University	NCT00672542	Metastatic melanoma	LMP2, LMP7, MECL1	Phase I
Tekmira Pharm.	TKM-ApoB	Hypercholesterolaemia	ApoB	Phase I
Zabecor	Excellair	Asthma	Syk	Phase II
Santaris Pharma	SPC3649	Hepatitis C virus	miR-122	Phase II

Table 2. RNAi-based compounds in clinical trials.

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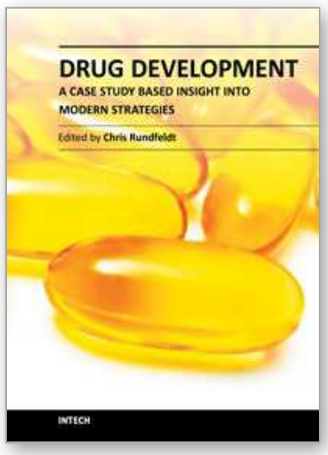

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This book represents a case study based overview of many different aspects of drug development, ranging from target identification and characterization to chemical optimization for efficacy and safety, as well as bioproduction of natural products utilizing for example lichen. In the last section, special aspects of the formal drug development process are discussed. Since drug development is a highly complex multidisciplinary process, case studies are an excellent tool to obtain insight in this field. While each chapter gives specific insight and may be read as an independent source of information, the whole book represents a unique collection of different facets giving insight in the complexity of drug development.

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