From DEPARTMENT OF LABORATORY MEDICINE
Karolinska Institutet, Stockholm, Sweden

DELIVERY OF GENE-REGULATING AGENTS: INTERNALIZATION MECHANISMS AND NOVEL VECTORS

Per Lundin

Karolinska Institutet

Stockholm 2011

Published by Karolinska Institutet. Printed by Universitetsservice AB.

ISBN 978-91-7457-432-6

To my family

ABSTRACT

The sequencing of the human genome was expected to generate a veritable explosion of therapeutics for regulation of gene function, either through modulation of gene expression or through the replacement of defect genes. However, nucleic acid-based pharmacological agents suffer from issues of low bioavailability and unfavorable pharmacokinetics, wherefore these prospects have not yet been realized.

One promising approach for regulation of gene function is a special type of antisense technology, referred to as splice correction. Aberrantly spliced mRNA is intimately associated with numerous serious illnesses, wherefore the ability to restore the correct splicing pattern is a highly attractive therapeutic approach. Another thriving oligonucleotide-based platform makes use of small (or short) interfering RNA (siRNA), double-stranded RNA sequences that efficiently silence expression of essentially any gene of interest. However, both platforms are limited by the inherent weaknesses of oligo- and polynucleotide-based agents, meaning that the development of efficient delivery vectors is a prerequisite for clinical translation. Short cationic peptide sequences, so called cell-penetrating peptides (CPPs), constitute an emerging category of delivery vehicles with the ability to convey various cargo molecules across the cell membrane, but numerous polymeric vectors (commonly referred to as 'polyplexes') are also under intense scrutiny for delivery of gene-regulating agents.

This thesis aims to delineate the internalization mechanisms of CPPs conjugated to a special type of splice-correcting oligonucleotide analogues (namely peptide nucleic acids (PNAs)), but it also presents a rationally modified CPP for delivery of splice-correcting oligonucleotides and plasmid DNA, as well as an entirely novel class of delivery vectors, so called polythiophenes, for siRNA delivery. Specifically, paper I examines the internalization routes of a number of CPP-PNA conjugates, papers II and III study the oligonucleotide and plasmid delivery efficacy, respectively, of the stearylated CPP transportan 10 (TP10), whereas paper IV examines the utility of a cationic polythiophene for siRNA delivery.

In conclusion, the research described herein provides novel data on internalization mechanisms of chemically distinct CPPs, as well as presents two novel agents for delivery of splice-correcting oligonucleotides, plasmid DNA, and siRNA, thereby adding additional tools to the toolbox for delivery of gene-regulating agents.

LIST OF PUBLICATIONS

I. Lundin, P., Johansson, H., Guterstam, P., Holm, T., Hansen, M., Langel, Ü., EL Andaloussi, S. Distinct uptake routes of cell-penetrating peptide conjugates. *Bioconjug Chem* 19, 2535-2542 (2008).

II. Mäe, M., EL Andaloussi, S., Lundin, P., Oskolkov, N., Johansson, HJ. J., Guterstam, P., Langel, Ü. A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation approach. *J Control Release* 134, 221-227 (2009).

III. Lehto, T., Simonson, O. E., Mäger, I., Ezzat, K., Sork, H., Copolovici, D. M., Viola, J. R., Zaghloul, E. M., Lundin, P., Moreno, P. M., Mäe, M., Oskolkov, N., Suhorutšenko, J., Smith C. I. E., EL Andaloussi, S. A peptide-based vector for efficient gene transfer in vitro and in vivo. *Mol Ther* 19, 1457-1467 (2011).

IV. Lundin, P., Viola, J. R., Shi, J., Smith, C. I. E, EL Andaloussi, S. (2011). Delivery of small interfering RNA (siRNA) using an amino acid-modified polythiophene. *Submitted*

ADDITIONAL PUBLICATIONS

V. Lundin, P. Is silence still golden? Mapping the RNAi patent landscape. *Nat Biotechnol* 29, 493-497 (2011).

VI. Lundin, P., Moreno, P. M., Smith, C. I. E., EL Andaloussi, S. Circular RNAi interference effector molecules (WO10084371). *Expert Opin Ther Pat* 21, 115-119 (2011).

VII. Lundin, P., EL Andaloussi, S., Langel, Ü. Toxicity methods for CPPs. *Methods Mol Biol* 683, 195-205 (2011).

VIII. EL Andaloussi, S., Lehto, T., Lundin, P., Langel, Ü. Application of PepFect peptides for the delivery of splice-correcting oligonucleotides. *Methods Mol Biol* 683, 361-373 (2011).

IX. Lundin, P. Clostridial translocation peptides for promoting endosomal escape of RNAi agents (WO09083738). *Expert Opin Ther Pat* 20, 975-980 (2010).

CONTENTS

LIST OF ABBREVIATIONS

2-*O*-Me RNA	2'-*O*-methyl RNA
Arg9	Nona-arginine
CIE	Clathrin-independent endocytosis
CME	Clathrin-mediated endocytosis
CPP	Cell-penetrating peptide
DNA	Deoxyribonucleic acid
GAG	Glucosaminoglycan
HIV	Human immunodeficiency virus
HS	Heparan sulfate
mRNA	Messenger RNA
miRNA	MicroRNA
PEI	Polyethyleneimine
Pen	Penetratin
PG	Proteoglycan
PNA	Peptide nucleic acid
POMT	Poly(3-[(*S*)-5-amino-5-methoxycarboxyl-3-oxapentyl]-2,5-thiophenylene hydrochloride)
PS	Phosphorothioate
RISC	RNA-induced silencing complex
RLU	Relative light (luminescence) units
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	Small (short) interfering RNA
SPPS	Solid-phase peptide synthesis
TP	Transportan

PREFACE

The main framework and the overall contents of this doctoral thesis draw heavily on research carried out at the Department of Neurochemistry, Stockholm University, summarized and published in the licentiate thesis *Cell-penetrating peptides in delivery of splice-correcting oligonucleotides* (Lundin, P., Stockholm University, Universitetsservice, Stockholm, 2008, ISBN 978-91-7155-774-2).

Specifically, paper I and paper II were present as manuscripts in the preceding thesis and the corresponding sections of the aims of the study, the methodological considerations, and the results and conclusions thus derive from this earlier publication. Furthermore, a substantial proportion of the introduction, in particular certain sections on cellular internalization mechanisms, antisense technology, alternative splicing, chemically modified nucleotides, and cell-penetrating peptides, originate from the licentiate thesis, although they have been revised and updated considerably to reflect the scientific progress over recent years.

1 INTRODUCTION

1.1 A CRUCIAL BARRIER

The human body is composed of trillions of cells, all with a specified function and with a distinct surrounding barrier. This barrier, the cell membrane, plays a number of fundamental roles for the cells' physiological functions; it is primarily a demarcating perimeter, separating the cell interior from the extracellular milieu and providing structural integrity, but it is also a point of contact, a scaffold for various types of macromolecules, and an entry barrier for most hydrophilic molecules. The membrane is primarily composed of phospholipids containing two separate domains, a polar head group and a hydrophobic fatty acid tail. As a result of the entropically driven hydrophobic effect these lipids form a virtually impermeable bilayer with a hydrophobic interior and a hydrophilic exterior.

The relatively recent advances within biotechnology, together with the sequencing of the human genome (Venter *et al*., 2001; IHGSC, 2001), was expected to generate a veritable explosion of new genetic therapies to combat serious illnesses, either through classical replacement of defect genes or through oligonucleotide-based modulation of gene expression. However, as a result of the poor pharmacokinetic profiles and the low bioavailability of nucleic acid-based compounds, primarily as an implication of the presence of the plasma (cell) membrane, only a very limited number of therapies have actually reached the bedsides. In order to enable transport of nucleic acid-based pharmacological agents into the cell interior, both non-viral (*i.e.* chemical and mechanical) and viral vectors have over the years been evaluated as delivery vehicles for gene-regulating oligonucleotides and for entire genes (in the form of plasmid DNA). Alas, these carriers generally suffer either from low efficacy or from issues of questionable patient safety (Somia & Verma, 2000).

Non-viral vectors normally share certain common denominators, such as positive charge and/or hydrophobicity, but the growing interest in drug delivery has generated a highly diverse flora of carrier compounds. One promising category of such non-viral delivery agents that has received increasing attention since their initial discovery in 1994 (Derossi *et al*., 1994) is the cell-penetrating peptides (CPPs). Despite their physical characteristics and the impermeability of the cell membrane to large, charged compounds, this group of highly hydrophilic molecules has the surprising ability not

only to translocate across the lipid bilayer but also to carry various cargo molecules inside the cell. Although the CPP field has been expanding significantly over recent years, the major delivery workhorses within nucleic acid contexts are cationic lipids (lipoplexes) and cationic polymers (polyplexes). The cationic polymer most commonly employed for gene therapy purposes is probably poly(ethylenimine) (PEI), a linear or branched amine-containing hydrocarbon chain, but numerous other polymers of different origins are constantly being developed.

The research presented in this thesis aims to devise strategies for traversing the entry barrier made up by the cell membrane, and the thesis spans the fields of cationic polymer- and CPP-mediated delivery of gene-regulating oligonucleotides and plasmid DNA, with specific foci on (i) the internalization mechanisms of a number of different CPPs conjugated to synthetic splice-correcting oligonucleotide analogues, as a means of delineating the endocytic internalization pathways of these short peptide vectors, (ii) development of a rationally designed chemically modified CPP with enhanced delivery properties, and (iii) the design of a novel cationic polymer vector for delivery of small interfering RNAs (siRNAs).

1.1.1 Cellular transport mechanisms

Despite its impermeability for large hydrophilic molecules, the plasma membrane does allow for vital small molecule-trafficking from the extracellular environment to the cytoplasm and other intracellular compartments. This type of membrane passage is based on either passive or active transport, mediated by specific membrane proteins, and it is limited to certain essential molecules.

For macromolecule and particle trafficking, cells utilize a different, multifaceted transport mechanism known as endocytosis. Specialized cells, primarily the leukocytes of the immune system, are capable of a specific type of large-particle endocytic process known as phagocytosis, whereas all body cells utilize the highly diverse process of pinocytosis for fluid-phase ingestion. The endocytosis research field is constantly evolving, making pathway classifications somewhat momentary and often tailored for specific scientific purposes. One relatively recent general categorization is based on the involvement of the vesicle-coating protein clathrin (Mayor & Pagano, 2007), giving two broad classes, namely clathrin-mediated (CME) and clathrin-independent (CIE) endocytosis (Figure 1). There are however separate pathways of immense cellular importance slightly peripheral to the normal endocytic

mechanisms, with perhaps the most prominent one being the bulk uptake process of macropinocytosis (Swanson, 2008).

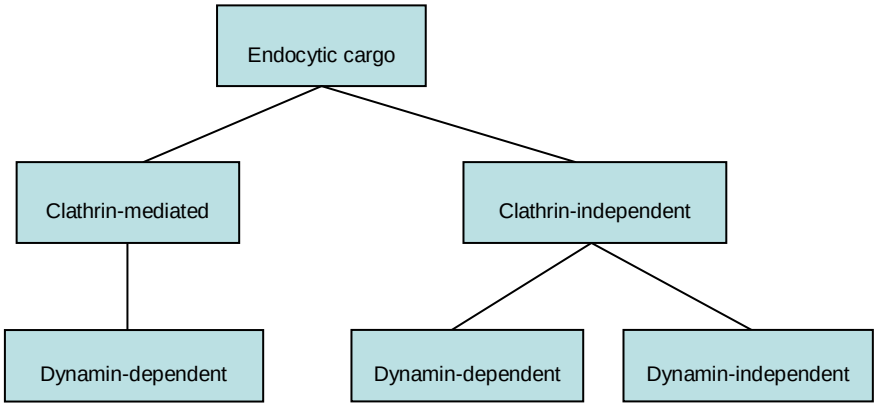

Figure 1. Recent endocytosis classification based on clathrin involvement, adapted from Mayor and Pagano (Mayor & Pagano, 2007).

1.1.2 Clathrin-mediated endocytosis

Clathrin, the coat protein behind the mechanistic endocytosis classification proposed by Mayor and Pagano, was first identified and isolated in the mid 1970s by the British cell biologist Barbara Pearse (Pearse, 1976). Adaptor protein recruitment of clathrin is the critical mechanism behind CME, a high-capacity process that occurs constitutively or in response to certain stimuli in all cell types as a pathway for internalization of, for instance, extracellular hormones and signalling factors (Takei & Haucke, 2001; Conner & Schmid, 2005; Howes *et al*., 2010). CME is intimately involved in various processes of crucial cellular importance, for instance neuronal synaptic vesicle retrieval (Girard *et al*., 2005), exo-endocytic receptor trafficking (Sheng & Kim, 2002), and neurotrophin uptake and sculpting of synaptic structure (Dickman, 2006).

CME is initiated by ligand-receptor interactions resulting in a concentration of activated receptors binding to cytosolic adaptor proteins, forming a link to a clathrin lattice. The clustering of receptors generates clathrin-coated, shallow membrane pits that subsequently invaginate, elongate and pinch off (Conner & Schmid, 2005; Collins *et al*., 2011). As can be deduced from Figure 1, CME is by default dependent on the 100 kDa GTPase dynamin (Mayor & Pagano, 2007). Dynamin is believed to mediate the excision of the 100-110 nm clathrin-coated vesicle from the plasma membrane (Takei *et al*., 1995; Swanson, 2008; Howes *et al*., 2010), generating a

vesicle for further cellular processing. It is worth noting that many accessory proteins play crucial roles in various functional aspects of CME: clathrin-associated sorting proteins (CLASPs) are involved in cargo selection; amphiphysins, endophilins, and epsins regulate coat maturation and invagination; and auxilin and synaptojanin are involved in vesicle uncoating (Jung & Haucke, 2007).

1.1.3 Clathrin-independent endocytosis

The clathrin-independent endocytic (CIE) pathways are not as extensively characterized as CME, but they are nevertheless broadly divided into a dynamin-independent and a dynamin-dependent pathway, each consisting of multiple processes (Mayor & Pagano, 2007).

One relatively well-characterized, dynamin-dependent CI pathway is caveolae- or raft-mediated endocytosis. This cholesterol-dependent pathway generates 50-80 nm flask-shaped invaginations enriched in cholesterol, sphingolipids, signalling proteins, and the cholesterol-binding hairpin-like integral membrane protein caveolin (Parton & Simmons, 2007). Endocytic cargoes include growth hormone receptors, cholera toxin subunit B, and viruses like SV40 (Parton & Richard, 2003). Another dynamin-dependent pathway is mediated by the GTPase RhoA and this mechanism, which leads to formation of 50-60 nm vesicles, is responsible for certain immune cell and fibroblast protein internalizations, for instance the uptake of the β-chain of the interleukin-2 receptor (Mayor & Pagano, 2007; Howes *et al*., 2010).

Many clathrin- and dynamin-independent pathways appear to have a common denominator in the involvement of small GTPases in the internalization mechanisms (Mayor & Pagano, 2007). The invaginations generated by these mechanisms often display rather distinctive morphological features, with long and wide surfaces surrounding a large volume of fluid (Sabharanjak *et al*., 2002).

1.1.4 Macropinocytosis

As the name implies, macropinocytosis belongs to the pinocytic internalization mechanisms but the pathway generates larger endocytic vesicles than commonly found within CME or CIE. In fact, macropinocytosis share a common feature with the large-scale uptake mechanism phagocytosis in that the Rho-family of GTPases triggers formation of actin-driven membrane protrusions (Conner & Schmid, 2005).

Macropinosome formation is either spontaneous (non-specific) or a response to growth factor-receptor stimulation from cell surface ruffles that close down and engulf extracellular fluids, forming vesicles varying in size from 0.2 to 10 µm (Swanson, 2008), but perhaps more commonly from 1 to 5 µm (Hillaireau & Couvreur, 2009).

The actin cytoskeleton movements are induced by the interactive effects of small (Ras-superfamily) GTPases and guanine exchange factors (GEFs) and results in flat sheet-like plasma membrane protrusions known as lamellipodia (Swanson, 2008). These structures either retract into the cytoplasm or close to form macropinosomes, a process that is, in certain cell types, induced by phosphatidyl-inositol 3-kinase (PI3K) (Araki *et al.*, 1996). One interesting aspect of the macropinocytic internalization process, which perhaps can be exploited in drug delivery, are the results pointing toward macropinosomes bypassing the lysosomal pathway, as suggested by Hewlett and colleagues (Hewlett *et al.*, 1994). Similarly, caveolae/raft-mediated endocytic processes appear not to generate acidified vesicles, but the resultant caveosomes have in fact been shown to display a neutral pH (Pelkmans & Helenius, 2002), meaning that the internalized delivery complex may fare better in caveosomes than in vesicular trafficking compartments resulting from other types of CIE or CME pathways. Although the exact intricacies of the various endocytosis pathways are still being investigated, it is clear that delivery vectors undergoing efficient endocytic internalization followed by endosomal escape could constitute an interesting platform for exploiting these endogenous uptake processes for drug delivery of compounds with low bioavailability, for instance nucleic acid-based material and proteins.

1.2 GENE REGULATION: OLIGONUCLEOTIDE-MEDIATED MODULATION AND GENE THERAPY

DNA, the genetic blueprint of all known living organisms, was first isolated almost one and a half decades ago, when the Swiss physician Friedrich Miescher was examining discarded surgical bandages (Miescher, 1871). The hereditary role of DNA was confirmed in the early 1950s, when Hershey and Chase showed that DNA was the genetic material of the T2 phage (Hershey & Chase, 1952), and the paramount breakthrough came just a few years later. Based on the X-ray diffraction images obtained by Rosalind Franklin (Franklin & Gosling, 1953), Watson and Crick

developed the first accurate model of the chemical structure of DNA (Watson & Crick, 1953), a discovery awarded with the Nobel Prize in Physiology or Medicine.

Half a century later, with the sequencing of the human genome and the concomitant mapping of numerous disease-causing genes (Venter *et al.*, 2001; IHGSC, 2001), the scene was set for rapid development of therapeutic agents for gene expression modulation. Together with the development of various tools for genetic interference, for instance the early demonstration in 1961 of hybridization-induced translation inhibition (Nireberg & Matthaei, 1961), the antisense proof-of-concept on Rous sarcoma virus (Stephenson & Zamecnik, 1978; Zamecnik & Stephenson, 1978), and the recent discovery of RNA interference (Fire *et al.*, 1998), the knowledge of the alphabet of the human genome was supposed to aid in combating numerous serious illnesses. However, transitioning basic research to the bedsides has proven to be difficult, and although the toolbox for gene regulation surely is a powerful one it remains to be seen when, and if, the promises of modulation of gene function will translate into clinical impact.

1.2.1 Antisense technology

Pharmacological agents based on different antisense platforms have been undergoing considerable preclinical and clinical investigation for various ailments, including Duchenne muscular dystrophy (Yin *et al.*, 2008; Goemans *et al.*, 2011), inflammatory diseases such as Crohn's disease (Yacyshyn *et al.*, 1998) and rheumatoid arthritis (Maksymowych *et al.*, 2002), oncology indications such as non-Hodgkin's lymphoma (Waters *et al.*, 2002), chronic lymphocytic leukemia (Dürig *et al.*, 2011), and various solid malignancies (Crooke, 2001), as well as viral infections (Cheng *et al.*, 1991). Antisense technology exploits the highly specific Watson-Crick hybridization of rather short oligonucleotides (approximately 15-25 nucleotides in length) to target RNA sequences in order to induce either enzymatic cleavage or steric obstruction of the ribosome, leading to translational arrest (Scherer & Rossi, 2003). There is a significant difference between these two approaches; when utilizing the antisense mechanism based on enzymatic cleavage, mediated by RNase H, the oligonucleotide concentration can be kept to a minimum as an implication of the multiple usage of the antisense agent, whereas the translational arrest strategy requires a vastly different stoichiometric relationship between the mRNA target and the therapeutic agent.

Despite the fact that the antisense paradigm was established already in the late 1970s only one single antisense-based drug (called Fomivirsen) currently has regulatory approval. Fomivirsen (marketed as Vitravene^{TM}) is a 21-mer phosphorothioate DNA oligonucleotide targeting viral mRNA corresponding to the coding region of the major immediate-early gene of human cytomegalovirus (CMV), and thereby inducing RNase H-mediated cleavage. Vitravene^{TM} is indicated for CMV-induced retinitis in immunocompromised patients and the drug is administered via intravitreal injection directly into the eye (Orr, 2001), thus bypassing the need for delivery vehicles, one of the aspects that has hampered drug development within the antisense field to date.

One special type of antisense platform that appears to be reaching the bedsides within a relatively near future, at least for certain severe neuromuscular indications (Goemans *et al*., 2011), makes use of so called splice-correcting oligonucleotides. The ability to correct splicing has relatively recently emerged as an interesting therapeutic approach, concomitantly with the realization that aberrant splicing is intimately associated with several diseases, such as cystic fibrosis and β-thalassaemia (Faustino & Cooper, 2003), as well as with several types of neoplasia (Pajares *et al*., 2007), and not least the debilitating disorders Duchenne muscular dystrophy (DMD) (Goemans *et al*., 2011) and spinal muscular dystrophy (Passini *et al*., 2011).

1.2.2 Alternative splicing and splice correction

As abovementioned, a specific type of antisense technology, based on splice-correcting oligonucleotides, is attracting increasing attention as evidence accumulates on the involvement of alternative splicing in various pathological processes. Aberrant splicing is not only involved in the abovementioned well-known ailments but several other disorders, such as Menke's disease (Madsen *et al*., 2008), ataxia-telangiectasia (Du *et al*., 2007), X-linked agammaglobulinemia (Ochs & Smith, 1996), and Hutchinson-Gilford Progeria Syndrom (Scaffidi & Misteli, 2005), originate in dysfunctional splicing processes.

The basis for the existence of alternative splicing is the presence of introns and exons in eukaryotic genes. Introns need to be removed prior to translation and this process is carried out by a sophisticated nuclear complex referred to as the spliceosome, comprising five types of small nuclear ribonucleoproteins (snRNPs) and hundreds of accessory proteins (Srebrow & Kornblihtt, 2006). On average, seven

splicing events occur per human pre-mRNA molecule, indicating the importance and critical nature of this process (Lander *et al*., 2001). The splicing mechanism is initiated when the spliceosome recognizes a 5' donor site beginning with a GU dinucleotide and a 3' acceptor site ending with an AG dinucleotide (in accordance with the co called "GU-AG rule") (Srebrow & Kornblihtt, 2006). Generally, the 5' site is identified by U1 snRNP and the 3' site by U2 snRNP, whereas the auxiliary spliceosome factor U2AF associates with a polypyrimidine tract on the pre-mRNA and facilitates correct U2 binding (Sharp, 1994; Douglas & Wood, 2011). With the recruitment of three additional snRNPs the excision of the intron is accomplished.

The main cause of alternative splicing is deviation from the consensus splice site sequence, generating a weak but functional site with lower affinity for the spliceosomal proteins and snRNPs (Srebrow & Kornblihtt, 2006). These sites are also influenced by so called enhancers and silencers, short stretches of approximately 10 nucleotides exerting either positive or negative effects on the splice sites through the binding of regulatory proteins (Graveley, 2000) or through silencer-mediated formation of pre-mRNA secondary structures (Buratti *et al*., 2004).

Approximately 15% of the mutations responsible for genetic disorders affect pre-mRNA splicing, either through disruption or generation of splice sites (Srebrow & Kornblihtt, 2006). The ability to correct aberrant splicing was first realized in 1993, when a splice-correcting oligonucleotide was designed to block an activated cryptic splice site in thalassemic β-globin pre-mRNA, thereby obstructing the binding of the spliceosome and restoring normal splicing (Dominski & Kole, 1993). Since then, splice correction has made great strides toward the bedsides and ongoing clinical activities hold great promise for the treatment of, for instance, Duchenne muscular dystrophy (Kinali *et al*., 2009; Goemans *et al*., 2011).

1.2.3 RNA interference

Although discovered only slightly more than a decade ago (Fire *et al*., 1998), RNA interference (RNAi), a mechanism for controlling gene expression found in many eukaryotic organisms, has turned into a veritable laboratory workhorse within numerous fields, and its pioneers were awarded the 2006 Nobel Prize in Physiology or Medicine for their work. Like the antisense strategies, RNAi relies on the complementarity between an interfering RNA and its target mRNA, resulting in a powerful specificity and a for many purposes pivotal target discrimination.

The RNAi-mediated gene silencing pathway is initiated by the interaction between long stretches of dsRNA and the RNase III-like endoribonuclease Dicer, an enzyme that digests the strands into short (20-30 nucleotides) double stranded oligonucleotides with 2-nucleotide 3' overhangs (Bernstein *et al*., 2001; Czech & Hannon, 2011). Subsequently, these short endogenous, foreign (*e.g.* viral), or synthetically introduced oligonucleotides enter a multiprotein structure referred to as the RNA-induced silencing complex (RISC) (Hammond *et al*., 2000). A heterodimer of two proteins, termed R2D2 and Dicer-2, unwinds the dsRNA and directs incorporation of a single strand (the "guide" strand (Sano *et al*., 2008)) into the mature RISC. The assembly of the cleavage-competent, mature RISC is achieved by the exchange of Dicer for an RNA-cleaving Ago-2 enzyme, together with incorporation of various accessory proteins followed by scanning of the mRNA pool for complementary sites and subsequent mRNA cleavage (van den Berg *et al*., 2008; Czech & Hannon, 2011).

The complementary of the RNA agent for the mRNA does not only convey unrivalled target specificity but it also mediates two distinct mechanisms depending on the degree of complementarity between the mRNA target and the seed region (*i. e*. the region responsible for target binding (Schwarz *et al*., 2006)) of the interfering RNA. Perfectly matching guide strands induce mRNA cleavage (in a 21-mer siRNA/mRNA duplex between positions 10 and 11 of the mRNA when counting from the 5' end of the antisense strand (Elbashir *et al*., 2001a)), whereas sequences with centrally bulging or imperfectly matching regions only promote translational inhibition (van den Berg *et al*., 2008).

As indicated above, the endogenous process of RNA interference can be exploited using synthetic double-stranded RNAs and there are several approaches for efficient silencing of gene expression. The most commonly utilized RNAs for the induction of RNAi are synthetic short interfering RNAs (siRNAs) (Elbashir *et al*., 2001b), commonly delivered by chemical means, and short hairpin RNAs (shRNAs) transcribed from a DNA construct (Rayburn & Zhang, 2008). The relatively short (20-22 nucleotides) siRNAs and shRNAs were developed to circumvent the mounting of an anti-viral interferon response upon introduction of longer stretches of double-stranded RNA (Rayburn & Zhang, 2008), but longer RNA stretches acting as Dicer substrates have been shown to enhance RNAi potency and efficacy (Kim *et al*., 2005).

MicroRNAs (miRNAs), an abundant class of endogenous transcripts involved in the RNAi machinery, are naturally occurring mammalian post-transcriptional gene regulatory elements (Ambros, 2001; Pratt & MacRae, 2009), suggested to account for a large number of diseases, including numerous malignancies (Georges *et al*., 2007; Jeyaseelan *et al*., 2007). These evolutionary conserved sequences, derived from non-coding regions, have been found in numerous species and their ability to reduce the level of their target transcripts is long-established, together with an involvement in such diverse and complex processes as cell proliferation control and brain patterning (Calin & Croce, 2006). miRNAs are generated from microprocessor complex-mediated cleavage of precursor transcripts, generating a short-hairpin pre-miRNA in the nucleus (Osada & Takahashi, 2007). After nuclear processing of pri-miRNA into pre-miRNA and subsequent export, pre-miRNAs are cleaved by Dicer/TRBP into (imperfectly matched) miRNA duplexes and the guide strand is incorporated into the RISC. The following gene silencing, which either relies on translational repression due to the formation of imperfect hybrids between the miRNA and the 3'-untranslated region (3' UTR) of the target mRNAs or on deadenylation and subsequent mRNA degradation (Fabian *et al*., 2010), is believed to regulate approximately 30% of the genes in the human genome (Osada & Takahashi, 2007).

RNAi has already proven itself extensively *in vitro* and several drug candidates based on RNAi are also currently under clinical investigation, with targets such as the vascular endothelial growth factor and its receptor as a therapy for age-related macular degeneration (AMD) (Davidson & McCray, 2011), the viral nucleocapsid of respiratory syncytial virus (RSV) (Vaishnaw et al., 2010), and silencing of ribonucleotide reductase as a treatment for cancer (Watts *et al*., 2008; Davis *et al*., 2010).

1.2.4 Gene transfer

The classical approach to gene therapy entails replacing a defect gene through the introduction of genetic material, frequently in the form of plasmid DNA (pDNA). Naturally, the size and polyanionic nature of plasmids imply that delivery vectors are an absolute necessity for most applications, although it has been shown that naked pDNA administered via intramuscular injection (i.m.) (Wolff *et al*., 1991), or via hydrodynamic injection in a particular therapeutic setting (Zhang *et al*., 2010), may

result in cellular uptake and gene expression (albeit at very modest levels when administered i.m.).

The natural capacity of viruses (*e.g.* adenoviruses, retroviruses, or lentiviruses (Waehler *et al.*, 2007)) to convey genetic material into host cells has traditionally been exploited in gene therapy settings, as a means to efficiently introduce a gene of interest into a target cell. Although a highly efficacious strategy, virus-mediated gene delivery may suffer from issues of questionable patient safety, for instance as an implication of the risk of transferring replication-competent viral particles, the risk of insertional mutagenesis (*i.e.* tumor induction), or the induction of severe immune reactions (Lundin *et al.*, 2009). Hence, even though the majority of clinical trials carried out within the gene therapy field so far has been exploiting viral vectors (Edelstein *et al.*, 2007), alternative, non-viral approaches for the transfer of genetic material in the form of pDNA have also been developed. These non-viral strategies can generally be divided into physical and chemical methods, but only chemical delivery vehicles will be discussed within the framework of this thesis.

1.2.5 Chemically modified oligonucleotides

Despite the specificity and versatility of oligonucleotide-mediated gene regulation as a therapeutic approach, the inherent characteristics of nucleic acid-based compounds are not optimal from a pharmacokinetic point of view. RNA, for instance, is highly vulnerable to nuclease activity (Behlke, 2008), leading to a half-life in serum of around 2 minutes for unmodified siRNA administered intravenously (Morris *et al.*, 2005). Furthermore, as a result of the polyanionic nature of oligonucleotides, their ability to cross cellular membranes is very limited. These electrostatic properties also lead to extensive hydration and reduced binding to serum proteins, resulting in rapid systemic clearance through the kidneys (Behlke, 2008; Watts *et al.*, 2008). Finally, dsRNA molecules have a propensity to elicit immune responses, primarily mediated by the innate immune system. The presence of dsRNA in the cytoplasm is often an indicator of viral infection, typically inducing an anti-viral type-1 interferon response (Schlee *et al.*, 2006; Robbins *et al.*, 2009). Immunorecognition of RNA depends on specific molecular characteristics, such as length, single- or double-strand, sequence motifs, and nucleoside modifications, and this recognition is mediated by three members of the Toll-like receptor (TLR) family, as well as by cytosolic RNA-binding proteins and helicases (Schlee *et al.*, 2006; Robbins *et al.*, 2009).

Synthetic oligonucleotides are often divided into first and second generation, depending on the backbone modification. One typical representative of first generation nucleic acid analogues is the phosphorothioate DNA, where the phosphodiester (PO) linkage has been replaced by a phosphorothioate (PS), which increases serum stability significantly and prolongs circulation times through its 'sticky' interaction with serum proteins (Spitzer & Eckstein, 1988; Campbell et al, 1990; Levin, 1999). Oligonucleotides of the second generation has 2'-*O* modifications, such as 2'-*O*-methyl or 2'-*O*-methoxethyl RNA, displaying greater affinity to RNA but, unlike first generation DNA analogues, do not recruit RNase H (Monia *et al*., 1993). Another commonly used synthetic analogue of the second generation is the peptide nucleic acid (PNA), a non-ionic, achiral, DNA analogue in which the PO backbone is replaced by 2-aminoethyl-glycine linkages (Nielsen *et al*., 1991). The nucleotide bases are attached to this backbone at the amino nitrogen, using a methyl carbonyl linker. These features render PNA resistant to chemical and enzymatic cleavage, it does not recruit RNase H, and its hybrid complexes exhibit extraordinary thermal stability (Ray & Nordén, 2000).

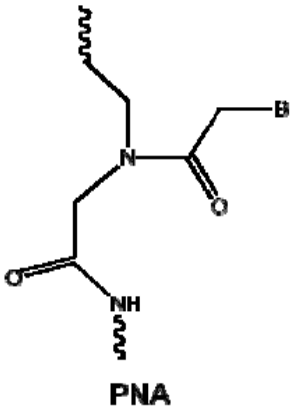

Figure 2. PNA

The ability of an oligonucleotide to engage in cellular pathways, be it through RISC-mediated cleavage or through RNase H recruitment, is an important parameter to take into account when selecting chemical modifications for different applications. When aiming to restore correct splicing, the recruitment of ribonucleases is naturally highly undesirable, since the mRNA is to remain intact after the splice-switching process. However, in for instance an RNAi setting, it is naturally crucial that any nucleotide modifications are introduced judiciously, in order not to interfere with the complex RISC-mediated silencing, and any synthetic nucleotide analogues should hence be selected accordingly.

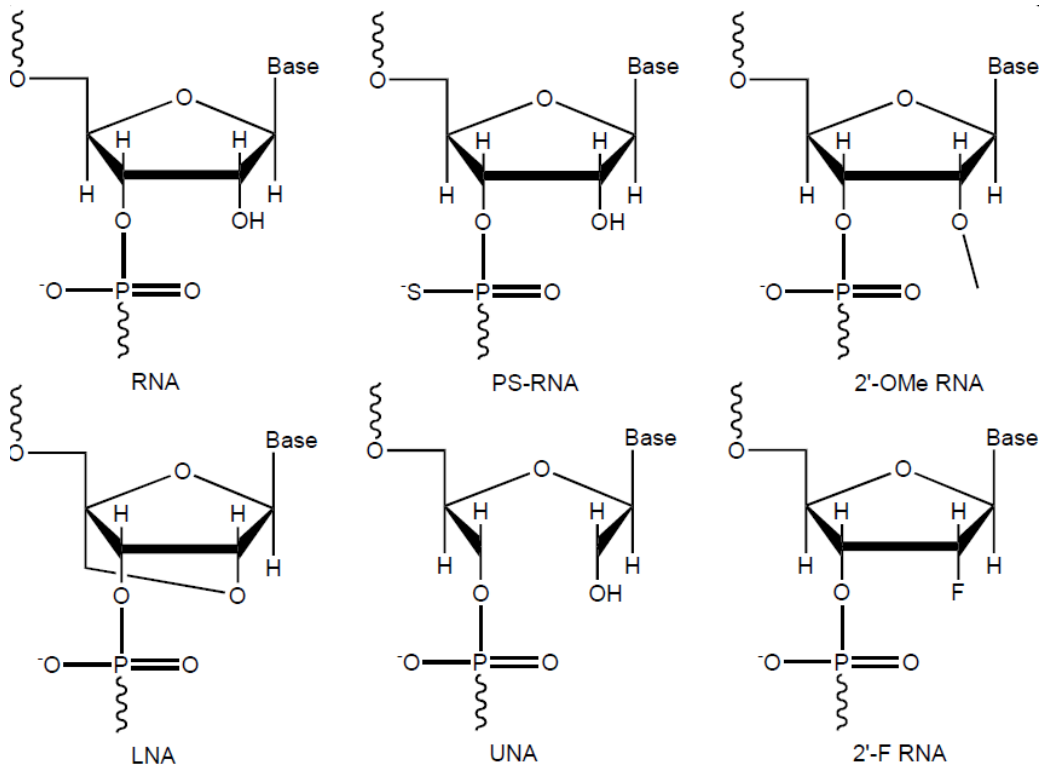

Figure 3. Commonly used RNA modifications. Native RNA is pictured on the top left. In the phosphorothioate (PS)-RNA a non-bridging oxygen atom is replaced by a sulfur atom; the 2'-O-methyl (2'-OMe) RNA is methylated on the 2'-OH moiety; the locked nucleic acid (LNA) has a methylene bridge linking the 2'-O and the 4'-C, locking the ribose ring into a 3'-C endo (North) conformation; the unlocked nucleic acid (UNA) lacks a bond between the 2'-C and the 3'-C atoms; and, in the fluoro (F)-RNA a fluorine atom is replacing the 2'-OH residue.

1.3 DELIVERY VECTORS

The low bioavailability and unfavorable pharmacokinetics of nucleic acid-based agents have spurred the development of a variety of different physical, chemical, and viral approaches for enhancing delivery. The physical methods, which rely on the application of a physical force to enable the oligo- or polynucleotide to penetrate the plasma membrane, include needle injection, gene gun, electroporation, and hydrodynamic delivery (extensively reviewed by Gao and colleagues (Gao *et al.*, 2007)), whereas the chemical means frequently involve, for instance, lipid-based material, cationic polymers, or peptide-based carriers (Wang *et al.*, 2010; Xu & Anchordoquy, 2011).

As discussed above in connection with gene transfer, the most efficient transfection approach is based on a biological system, namely viral vectors that infect the target cell. The major drawback of viral delivery stems from the potential

immunogenicity and risk for viral recombination (Gao *et al*., 2007), and furthermore, viral systems are not a viable option for transient delivery of short oligonucleotides. Although suitable for certain applications, viral delivery is, as is physical delivery methods, relatively laborious and as a result, numerous non-viral, chemical vectors are undergoing both preclinical and clinical evaluations in different settings. The two most commonly applied techniques for oligo- and polynucleotide delivery *in vitro* are based on the packaging of the nucleic acid by lipid-based agents or cationic polymers. Lipofection, *i. e*. non-covalent packaging of nucleic acids (*e.g.* DNA, as in the seminal publication) into small nuclease-protected liposomes, was first described by Felgner and colleagues in 1987 (Felgner *et al*., 1987), and this technique has been optimized and commercialized into mainstay transfection reagents for various purposes. Cationic liposomes and other forms of lipid-based materials facilitate endocytic cellular uptake and promote release from endosomal structures *in vitro* (Gao *et al*., 2007), and great strides have recently been made, perhaps primarily within the RNAi field, with lipid-based nanoparticles in preclinical and clinical trials against various therapeutic indications (Morrissey *et al*., 2005; Akinc *et al*., 2008; Abrams *et al*., 2010; Semple *et al*., 2010).

Cationic polymers constitute another class of commonly utilized transfection reagents for oligo- and polynucleotide delivery. Poly(ethyleneimine) (PEI) is probably the most extensively used polymer (Boussif, 1996), but it possesses the undesirable property of being non-biodegradable (Kunath *et al*., 2003). However, numerous biodegradable polymers, for instance chitosan (Lee & Mohapatra, 2008) and polylysine (Laemmli, 1975), are also being exploited for delivery of various types of nucleic acid-based agents, and the flora of polymer-based delivery vectors is constantly expanding.

A certain type of short, cationic or amphipathic peptides, belonging to a category termed cell-penetrating peptides (CPPs) or protein transduction domains (PTDs), are increasingly utilized for delivery of oligonucleotides and plasmids, both *in vitro* and *in vivo*. CPPs provide an efficient and often non-toxic way of transporting not only oligo- and polynucleotides but in fact most types of conceivable macromolecules into cells (EL Andaloussi *et al*., 2005).

1.3.1 Non-viral delivery agents

As an initial remark, it is worth noting that the determinants behind a successful non-viral delivery vector differ significantly depending on whether one is studying *in vitro* transfection of mammalian cells or *in vivo* delivery of a potential therapeutic agent, and, interestingly, there need not be any direct correlation between the two settings (Chang *et al*., 2007; Viola *et al*., 2009). Further divisions can also be identified within these two different contexts, with different cell lines (particularly adherent and suspension cell lines) placing different demands on a transfection reagent, and with for example different therapeutic indications, target tissues, therapeutic agents, and administration routes requiring different delivery vector properties *in vivo*.

Although highly variable, a few parameters are frequently discussed in the context of delivery of gene-regulating agents, namely nanoparticle size (suitably ranging from approximately 50 nm to 200 nm) and surface charge (often referred to as zeta (ζ) potential, a parameter that preferably ranges from -10 to + 10 mV) (Tong *et al*., 2009). The physical dimensions have obvious implications for aspects such as pharmacokinetics, internalization pathways, and biodistribution, whereas zeta potential is crucial for the colloidal stability of a nanoparticle suspension, as well as for both pharmacokinetic and pharmacodynamic behavior.

1.3.1.1. Cell-penetrating peptides (CPPs)

In the late 1980s it was realized that the Transactivator of transcription (Tat) protein of HIV-1 could translocate across the plasma membrane (Green & Loewenstein, 1988), and further studies revealed that merely a short stretch of amino acids was sufficient to promote membrane translocation (Vivés *et al*., 1997). In between these two discoveries, the group of Alain Prochaintz observed that the 60 amino acid homeodomain of the Antennapedia protein, from the fruit fly *Drosophila*, internalized into cells (Joliot *et al*., 1991), and that this activity was mediated by the third helix of the protein (Derossi *et al*., 1994). With this discovery, the CPP field was born and today numerous protein-derived as well as synthetic CPPs have been identified and designed.

The ability of CPPs to transport cargoes of various natures into cells has generated substantial interest from both a drug delivery perspective and from a more general research standpoint, and consequently CPPs are currently evaluated as delivery

vehicles for biologics, oligonucleotides, and small-molecule drugs, and utilized as research tools within various fields.

There is currently no exact definition of CPPs but they are generally below 30 amino acids in length and display a positive net charge at physiological pH, a feature which is important for the cell-penetrating properties. Most CPPs have none, or few, negative charges but the presence of acidic amino acids does not necessarily prevent internalization (Zorko & Langel, 2005). In addition to cationic amino acids, hydrophobic residues are found in most CPPs, perhaps with the exception of the homopolymeric polyarginine (Rothbard *et al*., 2000) and polylysine (Zhou *et al*., 1991). Cell-penetration has also been suggested to be intimately associated with structural polymorphism, *i. e*. the ability to change conformation in response to changes in the surrounding, for instance medium hydrophobicity or partner diversity (Deshayes *et al*., 2008; Konate *et al*., 2010). It has for instance been shown that monomorphic truncation analogues and mutants of the two commonly utilized CPPs penetratin and transportan (TP) display no cell-penetrating properties, indicating that structural flexibility may be a key component for efficient cell-penetration (Deshayes *et al*., 2008).

CPPs are commonly classified according to origin, with the three main categories being protein-derived, model, and designed CPPs (Zorko & Langel, 2005). Protein-derived CPPs are usually comprised of the minimal effective sequence of a translocation protein, whereas model peptides and designed CPPs generally originate from a rational design approach. Furthermore, CPPs can be classified based on their chemical properties, for instance in categories such as polycationic and primary or secondary amphipathic peptides (Howl *et al*., 2007). These categories naturally overlap, with the designed chimeric CPP transportan (Pooga *et al*., 1998) being a secondary amphipathic peptide, and the polycationic penetratin being, as mentioned above, protein-derived (Derossi *et al*., 1994).

Table 1. CPPs studied in this thesis.

CPP	Sequence	Reference
Protein-derived		
Penetratin	RQIKIWFQNRRMKWKK [a]	Derossi *et al.*, 1994
Tat (48-60)	GRKKRRQRRRPPQ	Vivés *et al.*, 1997
pVEC	LLIILRRRIRKQAHAHSK-amide	Elmquist *et al.*, 1998
M918	MVTVLFRRLRIRRASGPPRVRV-amide	EL Andaloussi *et al.*, 2007a
Designed/model peptides		
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-amide	Pooga *et al.*, 1998
TP10	AGYLLGKINLKALAALAKKIL-amide	Soomets *et al.*, 2001
MAP	KLALKLALKALKAALKLA-amide	Oehlke *et al.*, 1998
Arg9	RRRRRRRRR	Futaki *et al.*, 2001
MPG	GALFLGWLGAAGSTMGAPKKKRKV[b]	Morris *et al.*, 1997

Peptides are C-terminal free acids unless stated otherwise. [a] Originally with a free acid C-terminally but later shown also to have CPP properties when amidated. [b] C-terminal cysteamide group.

The internalization mechanisms of CPPs have caused great controversy, with the main dichotomy being whether the uptake is endocytic or not. It was previously commonly accepted that internalization did not involve endocytosis, until publications from separate groups revealed that conclusions regarding the translocation mechanisms could be based on artifacts in the experimental setup (Lundberg & Johansson, 2001; Richard *et al.*, 2003). Although some groups have been reporting on non-endocytic internalization for certain peptides, especially at high concentrations (Duchardt *et al.*, 2007; Kosuge *et al.*, 2008), even after the realization that the energy-independent internalization paradigm may have been flawed (Thorén *et al.*, 2003; Christiaens *et al.*, 2004; Duchardt *et al.*, 2007) the majority of studies are now emphasizing the importance of endocytosis in the uptake

of CPPs (Nakase *et al.*, 2004; Letoha *et al.*, 2005; EL Andaloussi *et al.*, 2007b). However, as an exception to the rule, in a recent report a secondary amphipathic peptide was shown to convey siRNA into a wide variety of cell lines via a seemingly endocytosis-independent pathway mediated by the interaction between aromatic tryptophan residues on the CPP and membrane components (Crombez *et al.*, 2009).

Most reports evaluating CPP uptake mechanisms have investigated the Tat peptide, either *per se* or conjugated to various cargo molecules (Fittipaldi *et al.*, 2003; Richard *et al.*, 2005). Internalization mechanisms of CPPs are generally assessed by employing different endocytosis inhibitors that act by selectively blocking particular endocytosis pathways, or through the use of fluorescently labeled pathway-specific tracers that are analyzed for co-localization with peptides. The results for the Tat peptide are divergent and, depending on study, the uptake has been ascribed classical clathrin-mediated endocytosis (CME) (Richard *et al.*, 2005), lipid raft/caveolae-mediated endocytosis (Fittipaldi *et al.*, 2003), macropinocytosis (Wadia *et al.*, 2004; Gump *et al.*, 2010), or a combination thereof (Duchardt *et al.*, 2007). This divergence can in part be explained by the fact that different cargo molecules have been used, indicating that the chemical nature of the entire complex influences the uptake mechanism, but internalization pathways may also vary with cell type and CPP concentration. Consequently, it comes as no surprise that different CPPs exploit different internalization mechanisms for uptake. A newly discovered CPP, termed M918, derived from the tumour suppressor protein p14ARF, has been shown to primarily exploit the macropinocytic pathway in internalization when conjugated to splice-correcting PNA (EL Andaloussi et al., 2007a), whereas, for instance, the chimeric transportan (TP) is believed to utilize clathrin-mediated endocytosis (CME) for internalization (Padari *et al.*, 2005).

The initial adherence of CPPs to cells is generally considered to be highly dependent on cell surface-anchored proteoglycans (PGs), predominantly glucosaminoglycans (GAGs) and particularly heparan sulfates (HS) (Poon & Gariépy, 2007). The high negative charge densities of the PGs mediate electrostatic interactions with the polycationic CPPs, a crucial event prior to endocytic internalization. GAG-deficient cells (Console *et al.*, 2003), and particularly cells lacking HS (Nakase *et al.*, 2007), exhibit a substantially decreased uptake, results that are further supported by the notion of PGs as accessory proteins in endocytosis (Belting, 2003). The picture is however far from clear, with a recent report showing that the Tat peptide traverses the cell membrane in a HS-independent manner,

whereas protease-catalyzed removal of cell surface proteins decreased Tat internalization considerably (Gump *et al*., 2010), indicating an important role played by membrane-anchored polypeptides as cellular interaction partners for CPPs.

The involvement of endocytosis in the uptake of CPPs have fuelled some concerns regarding the utility of these vectors in drug delivery (Foerg & Merkle, 2008) and endosomal release is often considered to be the rate-limiting step in CPP-mediated delivery (Wadia *et al*., 2004; Said Hassane *et al*., 2010). As an implication, numerous strategies have been developed in order to promote endosomal escape of CPP-based carriers, for instance the introduction of a lipid-destabilizing viral domain (Wadia *et al*., 2004), the design of fusogenic CPPs (Lundberg *et al*., 2007), and the rational incorporation of chloroquine-analogues into stearylated transportan 10 (EL Andaloussi *et al*., 2010).

CPPs have been utilized as delivery agents for various nucleic acid-based agents ever since the first example, an antisense oligonucleotide against amyloid precursor protein (APP), appeared in 1995 (Allinquant *et al*., 1995). Subsequently, CPPs have been exploited for delivery of, for instance, siRNA (Eguchi *et al*., 2009; EL Andaloussi *et al*., 2010) and splice-correcting oligonucleotides (Moulton *et al*., 2003; Yin *et al*., 2011), using either a non-covalent approach based on electrostatic and/or hydrophobic interactions (Simeoni *et al*., 2003; Crombez *et al*., 2009; EL Andaloussi *et al*., 2010) or covalent conjugation (Pooga *et al*., 1998; Bendifallah *et al*., 2006; Yin *et al*., 2011).

A number of novel, modified CPPs have relatively recently been introduced for the delivery of splice-correcting oligonucleotides with various nucleotide chemistries. Penetratin with an addition of six arginine residues (R_6-penetratin) and octaarginine repeatedly interrupted by the hydrophobic spacer 6-amino hexanoic acid have proved to promote efficient delivery of both PNA and morpholino oligonucleotides for splice correction (Abes *et al*., 2006; Abes *et al*., 2007). Not only is splice correction an appealing pharmaceutical platform, but as an implication of the nucleolar localization of the splicing machinery, delivery of splice-correcting oligonucleotides provides an elegant way of assessing CPP-mediated internalization in a functional context.

Table 2. CPP-delivered splice-correcting oligonucleotides.

CPP	Oligonucleotide	Pre-mRNA target	Reference
MAP	PNA	Luciferase	Wolf *et al*., 2006
$(RXR)_4$	Morpholino	Dystrophin	McClorey *et al*., 2006
R6-penetratin	Morpholino	Luciferase	Abes *et al*., 2007
Tat	PNA	Luciferase	Shiraishi *et al*., 2006
Pip2	PNA	Dystrophin	Ivanova *et al*., 2008

1.3.1.1 Cationic polymers

Due to the negative electrostatic potential of nucleic acid-based agents and of most synthetic nucleotide analogues (with the exception of peptide nucleic acids (PNAs) and phosphoroamidate morpholinos (PMOs)), chemical delivery vectors frequently display a net positive charge at physiological pH, in order to facilitate charge interaction with the polyanionic nucleic acid cargo. Cationic polymers commonly employed within gene transfer or in the delivery of synthetic oligonucleotides include poly(ethyleneimine) (PEI) (Boussif *et al*., 1995), cationic dendrimers (for instance poly(amidoamine)), 2-dimethyl(aminoethyl) methacrylate, or various forms of cationic polysaccharides such as chitosan or, as in the recent high-impact report on siRNA delivery, derivatized cyclodextrin (Davis *et al*., 2010).

The process for obtaining the nanoparticle-like delivery complexes (often termed 'polyplexes') generally involves condensing the nucleic acid cargo with the cationic polymer, using protocols of varying degree of complexity. Polyplex formation depends on a number of parameters, notably the condensation procedure *per se*, the ionic strength of the solution, the order of addition, and the charge and molar ratio of cationic polymer to nucleic acid cargo, and the resulting nanoparticles may consequently exhibit significantly different properties.

Complexation of plasmid DNA with, by way of illustration, the amine-containing hydrocarbon polymer poly(ethyleneimine) (Boussif *et al*., 1995), a mainstay *in vitro* transfection reagent, is for instance heavily influenced by the molecular weight of the

polymer, the so called nitrogen/phosphate (N/P) ratio (*i.e.* the ratio between nitrogen atoms on the PEI and nucleic acid phosphate groups), which in turn depends on whether the polymer is linear (Choosakoonkriang *et al.*, 2003) or branched (Gebhart & Kabanov, 2001), as well as the ionic strength of the formulation solution (Tros de Ilarduya *et al.*, 2010). PEI-mediated condensation, a process that is generally more efficient using PEI with high molecular weight and high charge density (Tros de Ilarduya *et al.*, 2010), decreases DNA volume considerably (Choosakoonkriang *et al.*, 2003) and the size of the resulting PEI/DNA nanoparticles may naturally be an important parameter behind delivery efficacy, both *in vitro* and *in vivo*.

In analogy with the highly diverse set of vectors categorized as CPPs, the plethora of cationic polymers used for delivery of gene-regulating agents appear to utilize different endocytic internalization processes as a way of conveying the nucleic acid cargo into the intracellular milieu. Although a positive surface charge has long been viewed as facilitating the interaction with the negatively charged proteoglycans on the cell surface (Behr *et al.*, 1989), the subsequent endocytic uptake pathways diverge, unsurprisingly, depending on aspects such as cell type and polymer characteristics, etc. (Tros de Ilarduya *et al.*, 2010). However, cationic polymers are commonly assumed to, one way or another, promote release of the nucleic acid cargo from endosomal compartments through the so called 'proton sponge' effect (Pollard *et al.*, 1998). The proton sponge effect essentially relies on the fact that the decreasing pH in endosomal compartments leads to a higher proportion of protonated nitrogen atoms in the cationic polymer, generating a charge gradient that induces an influx of chloride ions leading to osmotic swelling and finally endosomal rupture (Akinc *et al.*, 2005).

In order to further increase the utility of cationic polymers, including PEI, as nucleic acid delivery vehicles *in vivo*, covalent modification of the polymers with poly(ethyleneglycol) (PEG) is frequently employed, masking the often positive surface charge of the polyplexes. PEGylation of PEI has been shown to not only enhance stability of PEI/DNA complexes but also to reduce toxicity and significantly prolong circulation times, by decreasing interaction with blood components and non-specific accumulation in the liver after intravenous administration (Wolfert *et al.*, 1996; Sung *et al.*, 2003; Merdan *et al.*, 2005). PEG is also often included as a component in other types of delivery vectors, and the stealth properties it bestows to different vehicles have been crucial for the impressive existing preclinical and clinical pipeline of polymer and lipid-based delivery vectors (Morrissey *et al.*, 2005; Zimmerman *et al.*, 2006; Davis *et al.*, 2010).

Although stealth properties and favorable pharmacokinetics are important aspects for the *in vivo* performance of any type of delivery system it is sometimes necessary to utilize targeting strategies to attain relevant pharmacological effects. Targeting of a nano-sized delivery system to a cell type or tissue of interest can essentially be classified as either passive or active. Passive targeting relies on various manifestations of the underlying pathophysiology of the disease to be treated, with cancer being the textbook example, whereas active targeting is accomplished with the use of ligands interacting with a receptor on the target cell.

Solid cancer tumors exhibit tissue characteristics that are not observed in normal tissues or organs, namely defective vascular architecture, impaired lymphatic drainage, increased production of permeability mediators, such as various matrix metalloproteinases, and increased blood supply (Matsumura & Maeda, 1986; Maeda *et al*., 2000), leading to accumulation of nano-sized particles, for instance polyplexes and lipoplexes, in the tumor tissue. This phenomenon is termed the enhanced permeability and retention (EPR) effect and it implies that solid tumors are in fact passively targeted by most nanoparticle-like delivery vectors, a feature that can be exploited both for the delivery of gene-regulating agents and for chemotherapy delivery.

Active targeting, in contrast, entails incorporating a targeting ligand, with affinity for a receptor up-regulated on a cell type of interest, into a delivery vector. In a recent report on evidence of RNAi in humans from systemically administered siRNA-containing nanoparticles a transferrin ligand was utilized, in order to target the cyclodextrin-based vector to solid cancers refractory to standard-of-care therapies (Davis *et al*., 2010). However, the use of transferrin was pioneered in a polyplex context already back in 1997, when the group of Ernst Wagner covalently conjugated transferrin or an anti-CD3 antibody to PEI (Kircheis *et al*., 1997), thereby improving plasmid delivery considerably. Targeting ligands are nowadays a mainstay strategy for delivery of gene-regulating agents, and successful targeted polyplex delivery has been reported using for instance vitamins (notably folate (Hwa Kim *et al*., 2006)), peptides (Ke *et al*., 2009; Kim *et al*., 2011), and saccharides (Nie *et al*., 2010).

1.3.1.2 Lipid-based material

Lipid-based nucleic acid delivery vehicles have not been researched in any greater detail within the framework of this thesis, wherefore only a short account of their properties and applications will follow.

Liposome transfection was pioneered already back in 1987 (Felgner *et al.*, 1987) and today lipid-based platforms, comprising for instance a combination of an ionizable lipid, a neutral lipid, cholesterol, and a PEG lipid (Morrissey *et al.*, 2005; Zimmerman *et al.*, 2006), constitute state-of-the-art within the delivery of gene-regulating agents, particularly siRNAs (Morrissey *et al.*, 2005; Zimmerman *et al.*, 2006; Abrams *et al.*, 2010; Semple *et al.*, 2010).

Interestingly, capitalizing on the often modular nature of lipid-based delivery vehicles, the group of Robert Langer at MIT has adopted a combinatorial approach to developing lipid-based materials for delivery of gene-regulating agents, a strategy that has not only generated promising delivery data in various disease models but that has also resulted in proposed design criteria for lipid-based delivery materials (Akinc *et al.*, 2008; Frank-Kamenetsky *et al.*, 2008; Love *et al.*, 2010).

Though great strides have been made with lipid-based delivery vectors, particularly within the RNAi field where low doses of lipid-formulated siRNAs have resulted in sustained gene silencing (Akinc *et al.*, 2008; Frank-Kamenetsky *et al.*, 2008; Love *et al.*, 2010), their utility is to date almost exclusively limited to liver targeting, although at least one corporate player within the RNAi sector has been releasing information on successful siRNA delivery beyond the liver, notably to the vascular endothelium and to immune cells (Alnylam Pharmaceuticals, 2010). However, lipid-induced toxicity has been reported to constitute a significant hurdle for pharmaceutical development (Filion & Philips, 1998), meaning that there may be a bumpy road to clinical impact, despite the promising research presented to date.

2 AIMS OF THE STUDY

The research presented in this thesis spans the fields of cationic polymer- and CPP-mediated delivery of gene-regulating oligonucleotides and plasmid DNA, with specific foci on (i) internalization mechanisms of a number of different CPPs conjugated to synthetic splice-correcting oligonucleotide analogues, as a means of delineating the endocytic internalization pathways of these short peptide vectors, (ii) development of a rationally designed chemically modified CPP with enhanced delivery properties, and (iii) the design of a novel cationic polymer vector for delivery of small interfering RNAs (siRNAs). The main aims of the respective papers are presented below:

Paper I: The aim of this paper is to determine whether the delivery efficacy of conjugates between various CPPs and splice-correcting PNA correlates with the chemical nature of the CPP. Furthermore, the study is aiming at characterizing possible differences in internalization mechanisms of polycationic and amphipathic CPPs.

Paper II: The objective of this study is to investigate whether chemical modification, specifically stearylation, enhances CPP-mediated delivery of splice-correcting PS 2'-*O*-Me RNA oligonucleotides, using a non-covalent co-incubation approach.

Paper III: The aim of this paper is to evaluate the utility of the stearylated CPP TP10, identified in paper II, in the context of plasmid delivery *in vitro* and *in vivo*. Moreover, the study aims at characterizing the nanoparticles formed between stearylated TP10 and plasmid DNA (pDNA) from a physical perspective, as well as assessing the immunological impact, in cell culture and in mice, of the CPP/pDNA complexes.

Paper IV: The objective of this paper is the development and characterization of an entirely novel class of polymeric vectors, so called polythiophenes, for delivery of siRNA. Furthermore, the study intends to assess toxicological aspects relating to

the polythiophene-mediated siRNA delivery, as well as to shine light on possible internalization pathways.

3 METHODOLOGICAL CONSIDERATIONS

The methods utilized in this thesis are thoroughly described in each paper. Hence, merely some theoretical and practical aspects pertaining to methods and techniques of particular importance for the research will be discussed herein.

3.1.1 Solid-phase peptide and oligonucleotide synthesis

All peptides and PNAs used in this thesis were synthesized using solid-phase peptide synthesis (SPPS) methodology, a groundbreaking invention introduced by Bruce Merrifield in 1963 (Merrifield, 1963), resulting in paradigm shift within the peptide synthesis field. SPPS is based on repeated cycles of coupling and deprotection of protected amino acids, carried out on a solid support. Peptides and PNAs used in papers I and II were assembled by *t*-Boc chemistry using a 4-methylbenzhydrylamine-polysterene (MBHA) resin, generating C-terminally amidated peptides. The loading of resin for peptide synthesis was 1.16 mmol/g while the loading for PNA synthesis was substantially lower (0.1 mmol/g) as a result of the bulky nature of PNA monomers. Amino acids were coupled as hydroxybenzotriazole (HOBt) esters while PNA monomers were coupled with 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU).

Peptides and PNAs were cleaved from the resin using anhydrous hydrogen fluoride. To minimize side-reactions of reactive carbocations formed during cleavage, *p*-cresol, and if the peptide or PNA contained sulfur, *p*-thiocresol, was added to the cleavage mixture to act as scavengers. Following cleavage and extraction, peptides and PNAs were filtrated to remove the resin and subsequently freeze-dried. Crude products were purified using semi-preparative reversed-phase (RP) high performance liquid chromatography (HPLC) and analyzed using a matrix-assisted laser desorption/ionization-time of flight (MALDI–TOF) mass spectrometer (MS).

For covalent conjugation to PNA, peptides were synthesized with a cysteine residue having a 3-nitro-2-pyridinesulfenyl (Npys) group whereas the PNA contained a normal cysteine. Peptides and PNA were mixed in 20% acetonitrile/water, containing 0.1% trifluoroacetic acid (TFA), and stirred over night. Conjugates were purified and analyzed as described for peptides and PNAs.

The peptides studied in paper III were, in contrast, synthesized by fluorenylmethyloxycarbonyl (Fmoc) SPPS, using a Rink-amide methylbenzylhydrylamine resin to obtain C-terminally amidated peptides. Unlike *t*-Boc chemistry, Fmoc-based SPPS does not necessitate the use of hydrogen fluoride to cleave the peptides from the resin, wherefore Fmoc is generally considered a safer alternative. Again, peptides were purified using RP-HPLC and analyzed using MALDI–TOF MS.

Synthesis of PS 2-*O*-Me RNA was performed using disposable columns packed with polystyrene-based solid support, functionalized for synthesis of oligonucleotide sequences with 2-*O*-Me RNA monomers at the 3'-end. 5'-labelling was carried out using Cy5 amidite. Crude oligonucleotides were purified by anion exchange chromatography (AEC), desalted, and freeze-dried.

3.1.2 Covalent and non-covalent strategies for cargo attachment

In paper I, the splice-correcting PNA cargo was covalently coupled to the CPPs using a reducible disulfide bridge. The rationale behind utilizing this strategy is that the intracellular glutathione-mediated cleavage of the disulfide bond releases the cargo inside the cell (Hällbrink *et al.*, 2001), avoiding possible target interference. Additionally, this strategy produces well-defined chemical entities, which is advantageous for future potential therapeutic applications. However, the strategy is rather laborious, requiring extensive purification both prior to and after conjugation.

The non-covalent co-incubation strategy utilized in papers II-IV essentially only entails a simple mixing of the delivery vector and the nucleic acid cargo at a specified (molar or charge) ratio, resulting in the formation of nanoparticle complexes. The basis for the complex formation is believed to be predominantly reliant on electrostatic interactions between the polycationic agents and the polyanionic oligonucleotides, or oligonucleotide analogues, although it is likely that there is also a bond component originating from hydrophobic interactions. Naturally, an uncharged oligonucleotide, like PNA, is not compatible with the electrostatically driven co-incubation strategy, but the simplicity behind the co-incubation approach, in spite of the fact that it is not universally applicable, is nevertheless appealing. Furthermore, the co-incubation strategy generally requires smaller amounts of material than covalent conjugation, and the resulting nanoparticle complexes often display a high degree of serum stability.

3.1.3 Splice correction assay

In order to facilitate quantitative assessment of the cellular delivery efficiency of antisense oligonucleotides, the group of Ryszard Kole has developed a cellular splice reporter system (Kang *et al*., 1998) making it possible to assay for enzymatic activity instead of utilizing, for instance, more cumbersome PCR-based methods. The assay is based on HeLa cells stably transfected with a plasmid carrying a luciferase-encoding gene, interrupted by intron 2 from β-globin pre-mRNA. The intron contains an aberrant splice site that, unless it is masked by an antisense oligonucleotide, activates a cryptic splice site, generating non-functional luciferase (Figure 4). Consequently, detection of luminescence implies that the splice pattern has been modulated and this system is thus a useful indicator for both delivery efficacy and splice correction *per se*. In paper I, this assay was utilized to delineate uptake mechanisms of various CPPs, whereas in paper II, the assay verified the potency of the stearylated TP10 for non-covalent delivery.

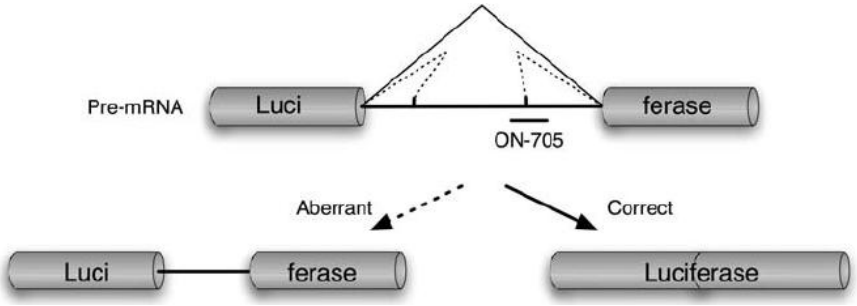

Figure 4. Pre-mRNA of the luciferase gene with the β-globin intron. Masking of the aberrant splice site is necessary in order to obtain functional luciferase.

3.1.4 Cell culture

The abovementioned cell line (HeLa pLuc 705) was utilized throughout all experiments in papers I and II of this thesis. HeLa cells derive from immortalized cervical cancer cells taken from a woman named Henrietta Lacks in the early 1950s. The cells are robust, grow rapidly, and are rather easy to transfect, making them a true medical and biological research workhorse over the past half a decade. In paper III, Chinese hamster ovary (CHO), HEK 293, U87, THP1, and U2OS cells were utilized,

together with mouse embryonal fibroblasts, whereas paper IV employed U2OS and HeLa cells.

3.1.5 Quantitative uptake measurements

Fluorescence-based measurements have traditionally been the mainstay approach for rapid CPP evaluation *in vitro*, both through the use of microscopy and through spectrofluorometry. The simplicity and rapid assay procedures are the appealing factors behind the widespread usage of fluorescence-based methods, but they are associated with certain drawbacks. When using spectrofluorometry it is virtually impossible to distinguish between peptides trapped in endosomal compartments and peptides inside the cell. As a consequence, the uptake of fluorophore-labeled peptides or polymers does not necessarily correlate with the bioactivity of the cargo molecule, for instance an oligonucleotide, wherefore it is pivotal to complement these assays with other methods.

The quantitative spectrofluorometry measurements utilized in this study are based on fluorescently labeled peptides or cargoes. The CPP constructs are incubated with cells for a certain time period, after which the cells are thoroughly washed, treated with trypsin, centrifuged, and lysed. Finally, fluorescence is measured and normalized to protein content in each well.

3.1.6 Proliferation and cytotoxicity measurements

Most CPPs generally exert limited toxic effects at the concentrations used in this study but polyplexes frequently cause toxicity, meaning that cytotoxicity assays are indispensable in order to avoid obtaining skewed results from other assays. Long-term cytotoxicity has been evaluated using, among other methods, the Wst-1 viability assay, which assesses proliferation by measuring mitochondrial metabolic activity, or more accurately the activity of mitochondrial dehydrogenases. Wst-1 is cleaved by the succinate-tetrazolium reductase system, producing the dye formazan, which can be spectrophotometrically quantified at 420 nm (Slater *et al.*, 1963). The conversion of Wst-1 is in linear correlation with the dehydrogenase activity and consequently the viability of the cells.

Monitoring the integrity of the plasma membrane is pivotal when utilizing CPPs or cationic polymers for delivery of various cargoes. If the membrane integrity has been

compromised, a typical sign of transient toxicity, lactate dehydrogenase (LDH), an enzyme catalyzing the conversion of lactate into pyruvate with simultaneous interconversion of NAD^+ into NADH, can leak out of the cell. Extracellular LDH-promoted NADH production can be detected via an enzymatic reaction where NADH reacts with reazurin and forms resorufin, which can be detected by measuring fluorescence at $560_{ex}/590_{em}$ nm. The LDH leakage assay provides a fast and simple assessment of plasma membrane integrity, but as LDH is a rather large protein the assay potentially fails to detect small pores created by a delivery vector.

4 RESULTS AND DISCUSSION

The first study presented herein utilizes CPP-mediated splice-correcting oligonucleotide delivery as an instrument for addressing internalization mechanisms of CPPs with different chemical properties. The second study presents a chemically modified CPP, stearylated transportan 10 (TP10), for highly efficient delivery of splice-correcting oligonucleotides using a non-covalent co-incubation strategy, whereas the third paper evaluates the utility of stearylated TP10 in pDNA delivery. Finally, the fourth study explores the utility for siRNA delivery of a novel class of polymeric vectors, known as polythiophenes.

4.1.1 CPP-mediated PNA uptake depends on the chemical nature of the CPP (Paper I)

The uptake mechanisms of CPPs are constantly debated, with different reports pointing in diverging directions. This study comprises an efficacy assessment and a mechanistic evaluation of seven commonly employed CPPs (Tat, penetratin, transportan (TP), transportan 10 (TP10), MAP, M918, and pVEC) using a splice-correcting PNA oligonucleotide cargo covalently conjugated to the peptides via a disulfide bridge. The CPPs originate from two different groups, displaying either a high degree of amphipathicity (TP, TP10, MAP, and pVEC) or a polycationic nature (Tat, penetratin, and M918).

No obvious correlation was observed between the chemical nature of CPPs and the delivery efficacy, as inferred from the results obtained with the previously described splice correction assay. The polycationic CPP Tat and the two amphipathic peptides pVEC and MAP were unable to promote significant delivery of the splice-correcting PNA, despite exhibiting a substantial quantitative uptake, thus indicating the necessity of using a functional assay when evaluating CPP delivery. Interestingly, clearly illustrating the discrepancy between uptake and bioactivity, the pVEC-PNA conjugate exhibited a very strong dose-dependent quantitative uptake but apparently failed to deliver the splice-correcting PNA to the nucleolus, where the spliceosome machinery performs its function.

Moreover, and perhaps slightly surprising at first glance, Tat, arguably the most commonly employed CPP, merely exhibited a very weak PNA delivery activity.

However, Tat is normally most efficient for delivery of polypeptide-based cargo molecules, wherefore its inability to efficiently transport a relatively small PNA appears quite logical. Hence it is important to bear in mind that different CPPs have different cargo preferences, meaning that the choice of vector must be governed by a careful assessment of the delivery properties of the CPP in light of the therapeutic application in question.

Further, the involvement of endocytosis was confirmed through the use of the lysosomotropic agent chloroquine (which exerts a 'proton sponge'-like effect on endosomes, thereby promoting endosomal release) and through measuring splice correction after treating cells at 4° C. As expected, chloroquine decreased the quantitative uptake as a result of its impeding effect on endocytosis, but the increased splice correction indicated release of material trapped in endosomal compartments. Treatment at low temperature also significantly diminished both quantitative uptake and splice correction, further corroborating the involvement of endocytic internalization processes.

In order to elucidate the endocytic mechanisms of the CPPs capable of successfully delivering the PNA cargo, a number of frequently used endocytosis inhibitors were employed. Chlorpromazine, an inhibitor of clathrin-mediated endocytosis (CME), significantly decreased the splice correction of the amphipathic peptide (TP and TP10) conjugates, whereas splice correction mediated by the polycationic penetratin and M918 was reduced when using the macropinocytosis inhibitors wortmannin and cytochalasin D. In addition, the ability of the CPPs to promote uptake of the fluid-phase markers dextran and transferrin further supported the role of distinct endocytosis pathways. M918 promoted significant dextran uptake, indicating that macropinocytosis is the main internalization mechanism, whereas TP10 induced transferrin uptake, confirming CME involvement.

The stipulated dependence on the extracellular proteoglycan heparan sulfate (HS) was assessed through pre-treatment of cells with the enzyme heparinase III, in order to remove HS from the cell surface. Splice correction decreased for all the conjugates, except M918-PNA, indicating that HS do play an important role prior to endocytic internalization. Interestingly, the splice correction mediated by M918 was in fact increased when applying heparinase treatment, perhaps suggesting involvement of another cell surface component that becomes more accessible when removing HS. As shown in a recent report, certain CPPs, specifically Tat (Gump *et al*., 2010), appear to require interaction with a polypeptide component prior to internalization, and one

could hypothesize that this is also the case for M918, which itself derives from the tumor suppressor protein p14ARF.

In conclusion, this study demonstrated low correlation between the ability to efficiently stimulate uptake of splice-correcting oligonucleotides and the chemical nature of the CPPs. However, the chemical characteristics of CPPs appeared to influence the employed endocytic internalization mechanism of the conjugates, providing additional insights into the parameters dictating CPP internalization.

4.1.2 A stearylated CPP for delivery of splice-correcting oligonucleotides (Paper II)

The non-covalent interaction between CPPs and negatively charged oligonucleotides is the fundamental factor behind the simplistic co-incubation strategy. Previously, two chemical modifications of CPPs, *i. e.* N-terminal stearylation and C-terminal cysteamidation, have been reported to be pivotal for efficient non-covalent delivery of oligonucleotides. In this study, N-terminal stearylation was evaluated for transportan 10 (TP10) and penetratin (Pen), and compared to the previously utilized stearylated nona-arginine (Arg9).

Unmodified CPPs co-incubated with phosphorothioate (PS) 2-*O*-methyl (2-*O*-Me) RNA did not promote any significant splice correction, despite displaying a substantial quantitative uptake. However, when utilizing the lysosomotropic agent chloroquine, the splice correction mediated by TP10-delivered PS 2-*O*-Me RNA increased dramatically, potentially indicating substantial endosomal entrapment of the PS 2-*O*-Me RNA/CPP complexes.

Introducing a C-terminal cysteamide improved the TP10-mediated transfection slightly, in fact to a level well above the transfection obtained with the previously used cysteamidated CPP MPG, but the increase was still rather modest. Consequently, in order to achieve more efficient transfection, all CPPs were N-terminally stearylated, and co-incubated with the splice-correcting oligonucleotide. Stearylated TP10 (TP10-stearyl) proved to be an extraordinarily efficient transfection agent, reaching splice correction levels in parity with the effects obtained with the commercially available transfection agent Lipofectamine 2000^{TM}. The quantitative uptake of TP10 increased slightly as an implication of stearylation, but the splice correction was increased by approximately a factor thirty, perhaps indicating that stearylation has a more substantial impact on endosomal escape than on uptake.

Interestingly, stearylation did not have such a pronounced effect on the polycationic CPPs penetratin and Arg9, suggesting that the interplay between the amphipathic TP10 and the stearyl moiety is crucial for endosomal escape.

The preserved efficiency in serum and the completely non-toxic behavior of TP10-stearyl are additional appealing features of this highly efficient delivery vector. These characteristics are essential for future *in vivo* applications, but further investigations are needed to verify its utility.

4.1.3 Stearylated TP10 promotes efficient *in vitro* and *in vivo* pDNA delivery (Paper III)

Encouraged by the efficacious oligonucleotide delivery promoted by the stearylated CPP TP10 in paper II, we set out, in this study, to evaluate its utility for the delivery of plasmid DNA, both *in vitro* and *in vivo*.

Again employing the non-covalent co-incubation approach, this study initially verified, using an ethidium bromide (EtBr) exclusion assay, that stearyl-TP10 had the ability to condense pDNA. Stearyl-TP10 promoted packaging of pDNA to a greater extent than its non-stearylated counterpart and the stability of the complexes, assessed using a heparin displacement assay, was considerably higher when using the stearylated TP10. The stearyl-TP10/plasmid nanoparticle complexes were subsequently studied using dynamic light scattering (DLS) and these measurements revealed, in accordance with the EtBr exclusion assay, that higher charge ratios (CRs) lead to the formation of nanoparticles with smaller dimensions. The overall size of the stearyl-TP10/plasmid nanoparticle complexes was in the range of 125-150 nm and the zeta (ζ) potential of the complexes was shown to range from approximately -5 to -11 mV.

In vitro experiments initially verified that unmodified CPPs (i.e. TP10 and Arg9) were unable to promote significant plasmid transfection in CHO cells, whilst stearyl-TP10 displayed considerable activity in CHO, U2OS, HEK293, and U87 cells, as well as in hard-to-transfect, primary mouse embryonal fibroblasts. A considerable decrease in transfection efficacy observed in glycosaminoglycan (GAG)-deficient CHO cells clearly pointed toward endocytosis involvement, and endocytic internalization was confirmed by co-treatment with the lysosomotropic agent chloroquine, which resulted in increasing transfection levels. In light of the condensation properties and the similarities between TP/plasmid and stearyl-TP10/plasmid in terms of quantitative uptake, as measured by spectroflourometry using fluorescently labeled peptides, this

indicates that the presence of the stearyl moiety on stearyl-TP10 most likely predominantly enhances delivery through nanoparticle stabilization and through promoting endosomal escape, and not by increasing cellular uptake.

In order to pave the way for *in vivo* evaluation, the toxicity profile and the immune-stimulatory effects of stearyl-TP10 were assessed using a cell viability assay and interleukin (IL) release measurements, respectively. Encouragingly, stearyl-TP10 did not exert any apparent toxic effects at any of the tested CRs and neither did the peptide induce release of IL-1β in cell culture or IL-6 *in vivo*, and based on these results the study was progressed into *in vivo* settings.

Balb/c mice were injected intramuscularly (i.m.) (into the *Musculus tibialis anterior*) and intradermally (i.d.) with stearyl-TP10/luciferase-encoding plasmid complexes at different CRs, and bioluminescence measurements were carried out at days 1, 3, 7, and 14, and at days 1, 3, and 7, respectively, post-injection. Only stearyl-TP10/pDNA at CR 1 promoted considerable luciferase expression upon i.m. and i.d. administration, but the luciferase expression displayed a dose-dependent increase over a range of from 1 to 10 µg of plasmid.

Taken together, stearyl-TP10 proved to be a widely applicable and efficacious transfection reagent for *in vitro* purposes and the fact that the peptide-based vector was capable of dose-dependently and sustainably increase gene expression *in vivo*, in combination with complete absence of systemic toxicity and immunogenicity, makes it an interesting delivery vector for future utilization, both *in vitro* and *in vivo*.

4.1.4 Polythiophenes as a novel class of siRNA delivery vectors (Paper IV)

The groundbreaking report of the utility of small interfering RNAs (siRNAs) for inducing RNA interference (RNAi) has initiated an extensive search for a magic delivery bullet. Although classical nucleic acid-delivery platforms (such as lipoplexes and polyplexes) are frequently employed also within the RNAi context numerous unconventional agents are being investigated as potential delivery vehicles. This study continues along those lines, utilizing a methylated serine-modified polythiophene known as POMT (poly(3-[(*S*)-5-amino-5-methoxycarboxyl-3-oxapentyl]-2,5-thiophenylene hydrochloride)), a compound to date predominantly used in biosensor applications, for the delivery of siRNAs.

POMT and its unmethylated, zwitterionic counterpart POWT (poly(3-[(S)-5-amino-5-carboxyl-3-oxapentyl]-2,5-thiophenylene hydrochloride)) exhibit substantial affinities for numerous biomolecules, particularly nucleic acids. Thus, through screening a range of molar ratios of an anti-luciferase siRNA to POMT/POWT in an *in vitro* assay, we set out to explore whether POMT and/or POWT could convey the siRNA cargo into the cytoplasm of a target cell. Both polythiophenes were in fact capable of promoting siRNA-induced silencing of the luciferase reporter gene in U2OS cells, but POMT displayed a considerable higher activity, reaching a maximum knockdown of approximately 85% using 50 nM siRNA at a molar ratio of siRNA:POMT of 1:50. POMT performed well also at lower siRNA concentrations (25 nm and 12.5 nM), and POMT-mediated delivery of an unrelated mock siRNA did not induce any statistically significant decrease in luminescence output at any evaluated molar ratio.

Nanoparticle tracking analysis (NTA) was subsequently used to assess the size of the siRNA:POMT complexes formed by co-incubating the two polyelectrolytes. A double-stranded DNA and a POMT analogue called tPOMT has previously been shown to form single, well-defined oligomolecular clusters at a molar ratio of DNA:tPOMT of 1:2, but in our setup, using molecules with a higher molecular weight as well as a higher molar ratio, we detected nanoparticle complexes having hydrodynamic diameters ranging from approximately 80 to 90 nm. The size of the siRNA:POMT complexes are in a suitable range for many therapeutic applications, notably cancer, since the nanoparticles are large enough to avoid first-pass glomerular clearance but small enough to diffuse into leaky tumor vasculature (Davis *et al*., 2008).

To further examine the interaction between siRNA and POMT, circular dichroism (CD) spectroscopy was used. The CD spectrum of POMT was indicative of a helical conformation, likely a function of the presence of supramolecular POMT clusters in which the individual polythiophenes are packaged so as to exhibit a helical structure. Upon addition of POMT to siRNA (at a molar ratio of 50:1), a slight decrease in signal intensity was observed, most likely originating from helicity-disturbing interspersion of siRNA with the POMT clusters, thereby confirming the presence of supramolecular siRNA:POMT aggregates or nanoparticles.

Through employing standard assays for endocytosis involvement, the internalization mechanism of the siRNA:POMT complexes was investigated. A significant decrease in gene silencing was seen when blocking energy-dependent

uptake, a result that indicated endocytic internalization, but pre-treatment with chloroquine did not, unexpectedly, induce a higher degree of knockdown. Taken together, these results point to endocytic internalization of the siRNA:POMT complexes but potentially also to efficient endosomal escape. In agreement with this hypothesis, live-cell fluorescence microscopy performed after transfection, exploiting the inherent optical properties of POMT and using a Cy5-labelled siRNA, detected a clear co-localization of siRNA and POMT, both in vesicular structures and in the cytoplasm.

Encouragingly, POMT did not exert any hemolytic effects in an *in vitro* assay, potentially indicating that the polythiophene-based delivery vector could be suitable for *in vivo* evaluation, albeit after further *in vitro* characterization and delineation of its internalization mechanism.

5 CONCLUSIONS AND FUTURE PROSPECTS

The main conclusions of the four papers comprising this thesis are presented below.

Paper I: The chemical nature of CPPs does not correlate with efficacy in terms of delivery of splice-correcting PNA-based oligonucleotides. However, polycationic CPPs appears to primarily utilize macropinocytosis for internalization whereas amphipathic CPPs exploit clathrin-mediated endocytosis (CME).

Paper II: The introduction of an N-terminal stearyl moiety on the amphipathic CPP transportan 10 (TP10) dramatically improves delivery of splice-correcting oligonucleotides, using a non-covalent co-incubation procedure. Furthermore, the potency is preserved in serum and the complex displays no cytotoxicity, making it a promising vector for future *in vivo* applications.

Paper III: The stearylated TP10 discovered in Paper II lends itself also to plasmid delivery, both *in vitro* and *in vivo*. The transfection efficacy of the stearyl-TP10/plasmid complexes reaches levels in parity with lipofection, but without any associated toxicity. Additionally, stearyl-TP10-mediated plasmid delivery results in sustained gene expression upon i.m. and i.d. injection into mice, warranting further *in vivo* evaluation.

Paper IV: The cationic polythiophene POMT, but not its zwitterionic counterpart POWT, shows great promise as an *in vitro* delivery vector for siRNA. Further, its complete absence of hemolytic activity could potentially render it suitable for *in vivo* siRNA delivery.

In conclusion, this thesis has provided new data regarding the internalization mechanisms of CPP conjugates, demonstrating that the chemical nature of the CPP appears to influence the endocytic internalization pathway. Furthermore, the thesis has introduced a new chemical entity, stearylated transportan 10, for efficient, non-toxic delivery of splice-correcting oligonucleotides *in vitro*, and pDNA both *in vitro* and *in vivo*. Additionally, the research described herein has lead to the identification of a novel use for the cationic polythiophene POMT in the delivery of siRNA,

potentially implying that polythiophenes as a class may be suitable for drug delivery purposes.

Even though the number of delivery vectors for nucleic acid-based agents has soared in recent years, the field appears to be in a seemingly never-ending search for a magic delivery bullet. In light of the multifaceted nature of the field this comes as no surprise, considering the numerous highly diverse therapeutic approaches to gene regulation (antisense, RNAi, splice-correction, and classical gene therapy, just to name a few), the almost endless number of 'druggable' targets, and thereby also the vast array of putative disease indications and administration modes. Hence, in spite of the plethora of existing delivery vectors for nucleic acid-based agents the non-viral delivery field has a clear *raison d'être*, as an implication of the ad hoc approach often needed in a specific therapy or disease context. Although ongoing clinical evaluations may provide certain indications as to what works and what does not in relevant settings it seems likely that the nucleic acid delivery field will continue to expand over the years to come, hopefully in parallel with widespread clinical translation of therapeutic platforms for gene regulation, and aided by the continued investigation into and development of novel delivery vectors.

6 ACKNOWLEDGEMENTS

The research described in this thesis has been carried out at the Unit for Molecular Cell Biology and Gene Therapy Science (MCG), Department of Laboratory Medicine, Karolinska Institutet (KI), and at the Department of Neurochemistry, Stockholm University (SU), and I am very much indebted to all my colleagues in both departments.

My time as a PhD student has been all but conventional and it would not have been possible for me to complete my studies without the help, flexibility, and support of a large number of people at the Karolinska Institute, at Stockholm University, but also at Albihns.Zacco AB, my employer over the last three years. I started my studies in the group of Ülo Langel at SU but after having been on a leave of absence in the corporate world I returned to academia to continue my PhD in the group of Ted Smith at KI. I would thus naturally like to express my gratitude to Ülo, but perhaps even more to Ted, who took me on halfway through my studies with unsurpassed flexibility and support.

The common denominator throughout my studies has been my good friend and main supervisor Samir EL Andaloussi, who initially introduced me to the mysteries of cell-penetrating peptides at SU and subsequently enabled me to continue my research at KI. Very few people can display the same type of excitement and encouragement for a new idea or for an interesting piece of result, and this attitude has been a constant source of inspiration during my studies.

I am also indebted to innumerable colleagues-turned-friends at SU, KI, and Albihns.Zacco. At SU I would especially like to highlight the friendship, support, and help of the oligo wiz Peter G and the Estonian squad comprising Indrek, Imre, and Maarja, at KI the Portuguese gang, and specifically my coworkers Joana and Pedro, and at Albihns.Zacco my lunch buddies Viktor and Jarkko. Additionally, I owe a lot to all the members of the MCG group for teaching me the drills in a new lab, for gladly sharing lab benches and equipment, and for just being a great bunch of people. I would also like to express a sincere thank you to all my friends outside work (not that many left after these years, perhaps!), in particular the KTH bunch (you know who you are!) and my good old friends Richard, Peter, Hanna, and Filip, for occasionally making it possible for me to forget about such trifling things as science and intellectual property.

Finally, my deepest gratitude goes to my parents, my sister Sofia, *farmor*, and my beloved girlfriend Nikki; words cannot begin to express how much you all mean to me. You are my world.

7 REFERENCES

Abes, S., Moulton, H. M., Clair, P., Prevot, P., Youngblood, D. S., Wu, R. P., Iversen, P. L., Lebleu, B. Vectorization of morpholino oligomers by the (R-Ahx-R)4 peptide allows efficient splicing correction in the absence of endosomolytic agents. *J Control Release* 116, 304-313 (2006).

Abes, S., Turner, J. J., Ivanova, G. D., Owen, D., Williams, D., Arzumanov, A., Clair, P., Lebleu, B. Efficient splicing correction by PNA conjugation to an R6-Penetratin delivery peptide. *Nucleic Acids Res* 35, 4495-4502 (2007).

Abrams, M. T., Koser, M. L., Seitzer, J., Williams, S. C., DiPietro, M. A., Wang, W., Shaw, A. W., Mao, X., Jadhav, V., Davide, J. P., Burke, P. A., Sachs, A. B., Stirdivant, S. M., Sepp-Lorenzino, L. Evaluation of efficacy, biodistribution, and inflammation for a potent siRNA nanoparticle: effect of dexamethasone cotreatment. *Mol Ther* 18, 171-80 (2010).

Akinc, A., Thomas, M., Klibanov, A. M., Langer, R. Exploring polyethyleneimine-mediated DNA transfection and the proton sponge hypothesis. *J Gene Med* 7, 657-663 (2005).

Akinc, A., Zumbuehl, A., Goldberg, M., Leshchiner, E. S., Busini, V., Hossain, N., Bacallado, S. A., Nguyen, D. N., Fuller, J., Alvarez, R., Borodovsky, A., Borland, T., Constien, R., de Fougerolles, A., Dorkin, J. R., Narayanannair Jayaprakash, K., Jayaraman, M., John, M., Koteliansky, V., Manoharan, M., Nechev, L., Qin, J., Racie, T., Raitcheva, D., Rajeev, K. G., Sah, D. W., Soutschek, J., Toudjarska, I., Vornlocher, H. P., Zimmermann, T. S., Langer, R., Anderson, D. G. A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat Biotechnol* 26, 561-569 (2008).

Allinquant, B., Hantraye, P., Mailleux, P, Moya, K., Bouillot, C., Prochiantz, A. Down-regulation of amyloid precursor protein inhibits neurite outgrowth in vitro. *J Cell Biol* 128, 919-927 (1995).

Alnylam Pharmaceuticals, Inc. Alnylam scientists presents new research on expanded delivery of RNAi therapeutics. <http://phx.corporate-ir.net/phoenix.zhtml?c=148005&p=irol-newsArticle2&ID=1480571&highlight=> (2010).

Ambros, V. microRNAs: tiny regulators with great potential. *Cell* 107, 823-826 (2001).

Araki, N., Johnson, M. T., Swanson, J. A. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. J Cell Biol 135, 1249-1260 (1996).

Belting, M. Heparan sulfate proteoglycan as a plasma membrane carrier. *Trends Biochem Sci* 28, 145-151 (2003).

Behlke, M. A. Chemical modification of siRNAs for in vivo use. *Oligonucleotides* 18, 305-320 (2008).

Behr, J.P., Demeneix, B., Loeffler, J.P., Perez-Mutul, J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine coated DNA. *Proc Natl Acad Sci U S A* 86, 6982–6986 (1989).

Bendifallah, N. Rasmussen, F. W., Zachar, V., Ebbesen, P., Nielsen, P. E., Koppelhus, U. Evaluation of cell-penetrating peptides (CPPs) as vehi-cles for intracellular delivery of antisense peptide nucleic acid (PNA). *Bioconjug Chem* 17, 750-758 (2006).

Bernstein, E., Caudy, A. A., Hammond, S. M., Hannon, G. J. Role for a bidentate ribonu-clease in the initiation step of RNA interference. *Nature* 409, 363-366 (2001).

Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* 92, 7297-7301 (1995).

Buratti, E., Muro, A. F., Giombi, M., Gherbassi, D., Iaconcig, A., Baralle, F. E. RNA folding affects the recruitment of SR proteins by mouse and human polypurinic enhancer elements in the fibronectin EDA exon. *Mol Cell Biol* 24, 1387-1400 (2004).

Calin, G. A., Croce, C. M. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* 66, 7390-7394 (2006).

Campbell, J.M., Bacon, T.A., Wickstrom, E. Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. *J Biochem Biophys Methods* 20, 259-267 (1990).

Chang, C. W., Choi, D., Kim, W. J., Yockman, J. W., Christensen, L. V., Kim, Y. H., Kim, S. W. Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. *J Control Release* 118, 245-253 (2006).

Cheng, Y., Gao, W., Han, F. Phosphorothioate oligonucleotides as potential antiviral compounds against human

immunodeficiency virus and herpes viruses. *Nucleosides and Nucleotides* 10, 155-166 (1991).

Choosakoonkriang, S., Lobo, B.A., Koe, G.S., Joe, J.G., Middaugh, C.R. Biophysical characterization of PEI/DNA complexes. *J Pharm Sci* 92, 1710–1722 (2003).

Christiaens, B., Grooten, J., Reusens, M., Joliot, A., Goethals, M., Vandekerckhove, J., Prochiantz, A., Rosseneu, M. Membrane interaction and cellular internalization of penetratin peptides. *Eur J Biochem* 271, 1187-1197 (2004).

Collins, A., Warrington, A., Taylor, K. A., Svitkina, T. Structural organization of the actin cytoskeleton at sites of clathrin-mediated endocytosis. *Curr Biol* 21, doi:10.1016/j.cub.2011.05.048 (2011).

Conner, S. D., Schmid, S. L. Regulated portals of entry into the cell. *Nature* 422, 37-44 (2005).

Console, S., Marty, C., Garcia-Echeverria, C., Schwendener, R., Ballmer-Hofer, K. An-tennapedia and HIV transactivator of transcription (TAT) "pro-tein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J Biol Chem* 278, 35109-35114 (2003).

Crombez, L., Aldrian-Herrada, G., Konate, K., Nguyen, Q. N., McMaster, G. K., Brasseur, R., Heitz, F., Divita, G. A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells. *Mol Ther* 1, 95-103 (2009).

Crooke, S. T., ed. Antisense Drug Technology: Principles, Strategies and Applications. New York: Marcel Dekker (2001).

Czech, B., Hannon, G. J. Small RNA sorting: matchmaking for Argonautes. *Nat Rev Genet* 12, 19-31 (2011).

Davidson, B. L., McCray, P. B. Jr. Current prospects for RNA interference-based therapies. *Nat Rev Genet* 12, 329-340 (2011).

Davis, M. E., Chen, Z., Shin, D. M. Nanoparticle therapeutics: an emerging treatment modality for cancer. *Nat Rev Drug Discov* 7, 771-782 (2008).

Davis, M. E., Zuckerman, J. E., Choi, C. H. J., Seligson, D., Tolcher, A., Alabi, C. A., Yen, Y., Heidel, J. D., Ribas, A. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* 464, 1067-1071 (2010).

Derossi, D., Joliot, A. H., Chassaing, G. H., Prochiantz, A. The third helix of the Anten-napdeia homeodomain translocates through biological membranes. *J Biol Chem* 269, 10444-10450 (1994).

Deshayes, S., Decaffmeyer, M., Brasseur, R., Thomas, A. Structural polymorphism of two CPP: an important parameter of activity. *Biochim Biophys Acta* 1778, 1197-1205 (2008).

Dickman, D. K., Lu, Z., Meinertzhagen, I. A., Schwarz, T. L. Altered synaptic development and active zone spacing in endocytosis mutants. *Curr Biol* 16, 591-598 (2006).

Dominski, Z., Kole, R. Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc Natl Acad Sci U S A* 90, 8673-8677 (1993).

Douglas, A. G. L., Wood, M. J. RNA splicing: disease and therapy. *Brief Funct Genomics* 10, 151-164 (2011).

Du, L., Pollard, J. M., Gatti, R. A. Correction of prototypic ATM splicing mutations and aberrant ATM function with antisense morpholino oligonucleotides. *Proc Natl Acad Sci U S A* 104, 6007-6012 (2007).

Duchardt, F., Fotin-Mleczek, M., Schwarz, H., Fischer, R., Brock, R. A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic* 8, 848-66 (2007).

Dürig, J., Dührsen, U., Klein-Hitpass, L., Worm, J., Hansen, J. B., Ørum, H., Wissenbach, M. The novel antisense Bcl-2 inhibitor SPC2996 causes rapid leukemic cell clearance and immune activation in chronic lymphocytic leukemia. *Leukemia* 4, 638-647 (2011).

Edelstein, M. L., Abedi, M. R., Wixon, J. Gene therapy clinical trials worldwide to 2007 – an update. *J Gene Med* 9, 833-842 (2007).

Eguchi, A., Meade, B. R., Chang, Y. C., Fredrickson, C. T., Willert, K., Puri, N., Dowdy, S. F. Efficient siRNA delivery into the primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. *Nat Biotechnol* 27, 567-571 (2009).

EL Andaloussi, S., Holm, T., Langel, Ü. Cell-penetrating peptides: mechanisms and applications. *Curr Pharm Des* 11, 3597-611 (2005).

EL Andaloussi, S., Johansson, H. J., Holm, T., Langel, Ü. A novel cell-penetrating peptide, M918, for efficient delivery of

proteins and peptide nucleic acids. *Mol Ther* 15, 1820-1826 (2007a).

EL Andaloussi, S., Johansson, H. J., Lundberg, P., Langel, Ü. Induction of splice correc-tion by cell-penetrating peptide nucleic acids. *J Gene Med* 8, 1262-1273 (2007b).

EL Andaloussi, S. E., Lehto, T., Mäger, I., Rosenthal-Aizman, K., Oprea, I. I., Simonson, O. E., Sork, H., Ezzat, K., Copolovici, D. M., Kurrikoff, K., Viola, J. R., Zaghloul, E. M., Sillard, R., Johansson, H. J., Said Hassane, F., Guterstam, P., Suhorutšenko, J., Moreno, P. M., Oskolkov, N., Hälldin, J., Tedebark, U., Metspalu, A., Lebleu, B., Lehtiö, J., Smith, C. I., Langel, U. Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. *Nucleic Acids Res* 39, 3972-3987 (2010).

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., Tuschl, T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J*, 20, 6877-6888 (2001a).

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494-498 (2001b).

Elmén, J., Lindow, M., Schütz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjärn, M., Hansen, H. F., Berger, U., Gullans, S., Kearney, P., Sarnow, P., Straarup, E. M., Kauppinen, S. LNA-mediated microRNA silencing in non-human primates. *Nature* 452, 896-899 (2008).

Elmquist, A., Lindgren, M., Bartfai, T., Langel, Ü. VE-cadherin-derived cell-penetrating peptide, pVEC, with carrier functions. *Exp Cell Res* 269, 237-244 (1998).

Fabian, M. R., Sonenberg, N., Filipowicz, W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* 79, 351-379 (2010).

Faustino, N. A., Cooper, T. A. Pre-mRNA splicing and human disease. *Genes Dev* 17, 419-437 (2003).

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., Danielsen, M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* 84, 7413-7417 (1987).

Filion, M. C., Philips, N. C. Major limitations in the use of cationic liposomes for DNA delivery. *Int J Pharm* 162, 159–170 (1998).

Fire, A., Xu, S., Montgomery, M. K., Kostas, S.A., Driver, S. E., Mello, C. C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391, 806-811 (1998).

Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F., Giacca, M. Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. *J Biol Chem* 278, 34141-34149 (2003).

Foerg, C., Merkle, H. P. On the biomedical promise of cell-penetrating peptides: limits versus prospects. *J Pharm Sci* 97, 144-162 (2008).

Frank-Kamenetsky, M., Grefhorst, A., Anderson, N. N., Racie, T. S., Bramlage, B., Akinc, A., Butler, D., Charisse, K., Dorkin, R., Fan, Y., Gamba-Vitalo, C., Hadwiger, P., Jayaraman, M., John, M., Jayaprakash, K. N., Maier, M., Nechev, L., Rajeev, K. G., Read, T., Röhl, I., Soutschek, J., Tan, P., Wong, J., Wang, G., Zimmermann, T., de Fougerolles, A., Vornlocher, H. P., Langer, R., Anderson, D. G., Manoharan, M., Koteliansky, V., Horton, J. D., Fitzgerald, K. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. *Proc Natl Acad Sci U S A* 105, 11915-11920 (2008).

Franklin, R. E., Gosling, R. G. Molecular Configuration in Sodium Thymonucleate. *Nature* 171, 740-741 (1953).

Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., Suguira, Y. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* 276, 5836-5840 (2001).

Gao, X., Kim, K. S., Liu, D. Nonviral gene delivery: what we know and what is next. *AAPS J* 9, 92-104 (2007).

Gebhart, C.L., Kabanov, A.V. Evaluation of polyplexes as gene transfer agents. *J Control Release* 73, 401–416 (2001).

Georges, M. Coppieters, W., Charlier, C. Polymorphic miRNA-mediated gene regulation: contribution to phenotypic variation and disease. *Curr Opin Genet Dev* 17, 166-176 (2007).

Girard, M., Allaire, P. D., McPherson, P. S., Blondeau, F. Non-stoichiometric relationship between clathrin heavy and light chains revealed by quantitative comparative proteomics of clathrin-coated vesicles from brain and liver. *Mol Cell Proteomics* 4, 1145-1154 (2005).

Goemans, N. M., Tulinius, M., van den Akker, J. T., Burm, B. E., Ekhart, P. F., Heuvelmans, N., Holling, T., Janson, A. A.,

Platenburg, G. J., Sipkens, J. A., Sitsen, J. M., Aartsma-Rus, A., van Ommen, G. J., Buyse, G., Darin, N., Verschuuren, J. J., Campion, G. V., de Kimpe, S. J., van Deutekom, J.C. Systemic administration of PRO051 in Duchenne's muscular dystrophy. *N Engl J Med* 16, 1513-1522 (2011).

Graveley, B. R. Sorting out the complexity of SR protein functions. *RNA* 6, 1197-1211 (2000).

Green, M., Loewenstein, P. M. Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* 55, 1179-1188 (1988).

Gump, J. M., June, R. K., Dowdy, S. F. Revised role of glycosaminoglycans in TAT protein transduction domain-mediated cellular transduction. *J Biol Chem* 285, 1500-1507 (2010).

Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 404, 293-6 (2000).

Hershey, A., Chase, M. Independent functions of viral protein and nucleic acid in growth of bacteriophage. *J Gen Physiol* 36, 39-56 (1952).

Hewlett, L. J., Prescott, A. R., Watts, C. The coated pit and macropinocytic pathways serve distinct endosome populations. *J Cell Biol* 124, 689-703 (1994).

Hillaireau, H., Couvreur, P. Nanocarriers' entry into the cell: relevance to drug delivery. *Cell Mol Life Sci* 66, 2873-2896 (2011).

Howes, M. T., Mayor, S., Parton, R. G. Molecules, mechanisms, and cellular roles of clathrin-independent endocytosis. *Curr Opin Cell Biol* 22, 519-527 (2010).

Howl, J., Nicholl, I. D., Jones, S. The many futures of cell penetrating peptides: how soon is now? *Biochem Soc Trans* 35, 767-769 (2007).

Hwa Kim, S., Hoon Jeong, J., Chul Cho, K., Wan Kim, S., Gwan Park, T. Target-specific gene silencing by siRNA plasmid DNA complexed with folate-modified polyethylenimine. *J Control Release* 104, 223-232 (2006).

Hällbrink, M., Florén, A., Elmquist, A., Pooga, M., Bartfai, T., Langel, Ü. Cargo delivery kinetics of cell-penetrating peptides. *Biochim Biophys Acta* 1515, 101-109 (2001).

International Human Genome Sequencing Consortium (IHGSC). Initial sequencing and analysis of the human genome. *Nature* 409, 860-921 (2001).

Ivanova, G. D., Arzumanov, A., Abes, R., Yin, H., Wood, M. J. A., Lebleu, B., Gait, M. J. Improved cell-penetrating peptide-PNA conjugates for splicing redirection in HeLa cells and exon skipping in mdx mouse muscle. *Nucleic Acids Res* 20, 6418-6428 (2008).

Jeyaseelan, K. Herath, W. B., Armugam, A. MicroRNAs as therapeutic targets in human diseases. *Expert Opin Ther Targets* 11, 1119-1129 (2007).

Joliot, A. H., Triller, A., Volovitch, M., Pernelle, C., Prochiantz, A. Alpha-2,8-polysialic acid is the neuronal surface receptor of antennapedia homeobox peptide. *New Biol* 3, 1121-1134 (1991).

Jung, N., Haucke, V. Clathrin-mediated endocytosis at synapses. *Traffic* 8, 1129-1136 (2007).

Kang, S. H., Cho, M. J., Kole, R. Up-regulation of luciferase gene expression with anti-sense oligonucleotides: implications and applications in functional assay development. *Biochemistry* 37, 6235-6239 (1998).

Ke, W., Shao, K., Huang, R., Han, L., Liu, Y., Li. J., Kuang, Y., Ye, L., Lou, J., Jiang, C. Gene delivery targeted to the brain using an Angiopep-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. *Biomaterials* 30, 6976-6985 (2009).

Kim, D-H., Behlke, M. A., Rose, S. D., Chang, M-S.Choi, S., Rossi, J. J. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* 23, 222-226 (2005).

Kim, J., Kim, S. W., Kim, W. J. PEI-g-PEG-RGD/small interference RNA polyplex-mediated silencing of vascular endothelial growth factor receptor and its potential as an anti-angiogenic tumor therapeutic strategy. *Oligonucleotides* 21, 101-107 (2011).

Kinali, M, Arechavala-Gomeza, V., Feng, L., Cirak, S., Hunt, D., Adkin, C., Guglieri, M., Ashton, E., Abbs, S., Nihoyannopoulos, P., Garralda, M. E., Rutherford, M., McCulley, C., Popplewell, L., Graham, I. R., Dickson, G., Wood, M. J., Wells, D. J., Wilton, S. D., Kole, R., Straub, V., Bushby, K., Sewry, C., Morgan, J. E., Muntoni, F. Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Ducenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *Lancet Neurol* 8, 918-928 (2009).

Kircheis, R., Kichler, A., Wallner, G., Kursa, M., Ogris, M., Felzmann, T., Buchberger, M., Wagner, E. Coupling of cell-binding

ligands to polyethylenimine for targeted gene delivery. *Gen Ther* 4, 409-418 (1997).

Konate, K., Crombez, L., Deshayes, S., Decaffmeyer, M., Thomas, A., Brasseur, R., Aldrian, G., Heitz, F., Divita, G. Insight into the cellular uptake mechanism of a secondary amphipathic cell-penetrating peptide for siRNA delivery. *Biochemistry* 49, 3393-3402 (2010).

Kosuge, M., Takeuchi, T., Nakase, I., Jones, A. T., Futaki, S. Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, plasma membrane associated proteoglycans. *Bioconjug Chem.*, 19, 656-664 (2008).

Kunath, K., von Harpe, A., Fischer, D., Petersen, H., Bickel, U., Viogt, K., Kissel, T. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J Control Release* 89, 113-125 (2003).

Laemmli, U. K. Characterization of DNA condensates induced by polyethylene(oxide) and polylysine. *Proc Natl Acad Sci U S A* 72, 4288-4292 (1975).

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. et al. Initial sequencing and analysis of the human genome. *Nature* 409, 860-921 (2001).

Lee, D., Mohapatra, S. S. Chitosan nanoparticle-mediated gene transfer. *Methods Mol Biol* 433, 127-140 (2008).

Letoha, T., Gaal, S., Somlai, C., Venkei, Z., Glavinas, H., Kusz, E., Duda, E., Czaljik, A., Petak, F., Penke, B. Investigation of penetratin peptides. Part 2. In vitro uptake of penetratin and two of its derivatives. *J Pept Sci* 11, 805-811 (2005).

Levin, A. A. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim Biophys Acta* 1489, 69-84 (1999).

Lingor, P., Koeberle, P., Kügler, S., Bähr, M. Down-regulation of apoptosis mediators by RNAi inhibits axotomy-induced retinal ganglion cell death in vivo. *Brain* 128, 550-558 (2001).

Love, K. T., Mahon, K. P., Levins, C. G., Whitehead, K. A., Querbes, W., Dorkin, J. R., Qin, J., Cantley, W., Qin, L. L., Racie, T., Frank-Kamenetsky, M., Yip, K. N., Alvarez, R., Sah, D. W., de Fougerolles, A., Fitzgerald, K., Koteliansky, V., Akinc, A., Langer, R., Anderson, D. G. Lipid-like material for low dose, in vivo gene silencing. *Proc Natl Acad Sci U S A* 107, 1864-1869 (2010).

Lundberg, M., Johansson, M. Is VP22 nuclear homing an artifact? *Nat Biotechnol* 19, 713-714 (2001).

Lundberg, P., EL Andaloussi, S., Sütlü, T., Johansson, H., Langel, Ü. Delivery of short interfering RNA using endosomolytic cell-penetrating peptides. *FASEB J* 21, 2664-2671 (2007).

Lundin, K. E., Simonson, O. E., Moreno, P. M. D., Zaghloul, E. M., Oprea, I. I., Svahn, M. G., Smith, C. I. E. Nanotechnology approaches for gene transfer. *Genetica* 137, 47-56 (2009).

Madsen, E. C., Morcos, P. A., Mendelsohn, B. A., Gitlin, J. D. In vivo correction of a Menkes disease model using antisense oligonucleotides. *Proc Natl Acad Sci U S A* 105, 3909-3914 (2008).

Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release* 65, 271-284 (2000).

Maksymowych, W., Blackburn, W., Tami, J.A., Shanahan, W. R. A pilot study of ISIS 2302, an antisense oligodeoxynucleotide, in patients with active rheumatoid arthritis. *J Rheumatol* 29, 447-53 (2002).

Matsumura, Y., Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 46, 6387-6392 (1986).

Mayor, S. Pagano, R. E. Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol* 8, 603-612 (2007).

McClorey, G., Moulton, H.M., Iversen, P.L., Fletcher, S., Wilton, S.D. Antisense oli-gonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. *Gene Ther* 13, 1373-81 (2006).

Merdan, T., Kunath, K., Petersen, H., Bakowsky, U., Voigt, K. H., Kopecek, J., Kissel, T. PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice. *Bioconjug Chem* 16, 785-792 (2005).

Merrifield, R.B. Solid Phase Peptide Synthesis. 1. The synthesis of a tetrapeptide. *J Am Chem Soc* 85, 2149-2154 (1963).

Miescher, F. Ueber die chemische Zusammensetzung der Eiterzellen. *Med-Chem* 4, 441-460 (1871).

Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., Freier, S. M. Evaluation of 2'-modified oligonucleotides con-taining 2'-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* 268, 14514-22 (1993).

Morris, M.C., Vidal, P., Chaloin, L., Heitz, F., Divita, G. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res* 25, 2730-6 (1997).

Morrisey, D.V., Lockridge, J.A., Shaw, L., Blanchard, K., Jensen, K., Breen, W., Hartsough, K., Machemer, L., Radka, S., Jadhav, V., Vaish, N., Zinnen, S., Vargeese, C., Bowman, K., Shaffer, C.S., Jeffs, L.B., Judge, A., Maclachlan, I., and Polisky, B. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol* 23, 1002–1007 (2005).

Moulton, H.M., Hase, M.C., Smith, K.M., Iversen, P.L. HIV Tat peptide enhances cellular delivery of antisense morpholino oligomers. *Antisense Nucleic Acid Drug Dev* 13, 31-43 (2003).

Nakase, I., Niwa, M., Takeuchi, T., Sonomura, K., Kawabata, N., Koike, Y., Takehashi, M., Tanaka, S. Ueda, K., Simpson, J. C., Jones, A. T., Sugiura, Y. Futaki, S. Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol Ther* 10, 1011-1022 (2004).

Nakase, I., Tadokoro, A., Kawabata, N., Takeuchi, T., Katoh, H., Hiramoto, K., Negishi, M., Nomizu, M., Sugiura, Y., Futaki, S. Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. *Biochemistry* 46, 492-501 (2007).

Nie, C., Liu, C., Chen, G., Dai, J., Li, H., Shuai, X. Hepatocyte-targeted psiRNA delivery mediated by galactosylated poly(ethylene glycol)-graft-polyethylenimine in vitro. *J Biomater Appl* May 28 [Epub ahead of print] (2010).

Nielsen, P. E., Egholm, M., Berg, R. H., Buchardt, O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 254, 1497-1500 (1991).

Nirenberg, M. W., Matthaei, J. H. The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotide. *Proc Natl Acad Sci U S A* 47, 1588-1602 (1961).

Ochs, H. D., Smith, C. I. X-linked agammaglobulinemia. A clinical and molecular analysis. *Medicine (Baltimore)* 75, 287-299 (1996).

Oehlke, J., Scheller, A., Wiesner, B., Krause, E., Beyermann, M., Klauschenz, E., Melzig, M., Bienert, M. Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically. *Biochim Biophys Acta* 1414, 127-139 (1998).

Orr, R. M. Technology evaluation: fomivirsen, Isis Pharmaceuticals Inc/CIBA vision. *Curr Opin Mol Ther* 3, 288-294 (2001).

Osada, H., Takahashi, T. MicroRNAs in biological process and carcinogenesis. *Carcinogenesis* 28, 2-12 (2007).

Padari, K., Säälik, P., Hansen, M., Koppel, K., Raid, R., Langel, Ü. Cell transduction pathways of transportans. *Bioconjug Chem* 16, 1399-1410 (2005).

Pajares, M. J., Ezponda, T., Catena, R., Calvo, A., Pio, R., Montuenga, L. M. Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncol* 8, 349-357 (2007).

Parton, R. G., Richards, A. A. Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. *Traffic* 4, 724-738 (2003).

Parton, R. G., Simmons, K. The multiple faces of caveolae. *Nat Rev Mol Cell Biol* 8, 185-194 (2007).

Passini, M. A., Bu, J., Richards, A. M., Kinnecom, C., Sardi, S. P., Stanek, L. M., Hua, Y., Rigo, F., Matson, J., Hung, G., Kaye, E. M., Shihabuddin, L. S., Krainer, A. R., Bennett, C. F. Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci Transl Med* 3, 72ra18 (2011).

Pearse, B. M. Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc Natl Acad Sci U S A* 73, 1255-1259 (1976).

Pelkmans, L., Helenius, A. Endocytosis via caveolae. *Traffic* 3, 311-320 (2002).

Pollard, H., Remy, J. S., Loussouarn, G., Demolombe, S., Behr, J. P. Escande, D. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J Biol Chem* 273, 7507–7511 (1998).

Pooga, M., Hällbrink, M., Zorko, M., Langel, Ü. Cell penetration by transportan. *FASEB J* 12, 67-77 (1998).

Pooga, M., Soomets, U., Hällbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J. X., Xu, X. J., Wiesenfeld-Hallin, Z., Hökfeldt, T., Bartfai, T., Langel, Ü. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain

transmission in vivo. *Nat Biotechnol* 16, 857-861 (1998).

Poon, G. M. K., Gariépy, J. Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. *Biochem Soc Trans* 35, 788-793 (2007).

Pratt, A. J., MacRae, I. J. The RNA-induced silencing complex: A versatile gene silencing machine. *J Biol Chem* 284, 17897-17901 (2009).

Ray, A., Nordén, B. Peptide nucleic acid (PNA): its medical and biotechnological applica-tions and promise for the future. *FASEB J* 14, 1041-1060 (2000).

Rayburn, E. R., Zhang, R. Antisense, RNAi, and gene silencing strategies for therapy: Mission possible or impossible. *Drug Discov Today* 13, 513-521 (2008).

Richard, J. P., Melikov, K., Brooks, H., Prevot, P., Lebleu, B., Chernomordik, L. V. Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. *J Biol Chem* 280, 15300-15306 (2005).

Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., Lebleu, B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* 278, 585-590 (2003).

Robbins, M., Judge, A., Maclachlan, I. siRNA and innate immunity. *Oligonucleotides* 19, 89-101 (2009).

Rothbard, J. B., Garlington, S., Lin, Q., Kirschberg, T., Kreider, E., McGrane, P. L., Wender, P.A., Khavari.,P. A. Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. *Nat Med* 6, 1253- 1257 (2000).

Sabharanjak, S., Sharma, P., Parton, R. G., Mayor, S. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell* 2, 411-423 (2002).

Said Hassane, F., Saleh, A. F., Abes, R. Gait, M. J., Lebleu, B. Cell penetrating peptides: overview and applications to the delivery of oligonucleotides. *Cell Mol Life Sci* 67, 715-26 (2010).

Sano, M., Sierant, M., Miyagishi, M., Nakanishi, Takagi, Y., Sutou, S. Effect of asymmet-ric terminal structures of short RNA duplexes on the RNA interference activity and strand selection. *Nucleic Acids Res* 18, 5812-5821 (2008).

Scaffidi, P., Misteli, T. Reversal of the cellular phenotype in the premature ageing disease Hutchinson-Gilford Progeria Syndrom. *Nat Med* 11, 440-445 (2005).

Scherer, L. J., Rossi, J. J. Approaches for the sequence specific knockdown of mRNA. *Nat Biotechnol* 21, 1457-1465 (2003).

Schlee, M., Hornung, V., Hartmann, G. siRNA and isRNA: two edges of one sword. *Mol Ther* 14, 463-470 (2006).

Schwarz, D. S., Ding, H., Kennington, L, Moore, J. T., Schelter, J., Burchard, J., Linsley, P. S., Aronin, N., Xu, Z., Zamore, P. D. Designing siRNA that distinguish between genes that differ by a single nucleotide. *PLoS Genet* 2, e140 (2006).

Semple, S.C., Akinc, A., Chen, J., Sandhu, A. P., Mui, B. L., Cho, C. K., Sah, D. W., Stebbing, D., Crosley, E. J., Yaworski, E., Hafez, I. M., Dorkin, J. R., Qin, J., Lam, K., Rajeev, K. G., Wong, K. F., Jeffs, L. B., Nechev, L., Eisenhardt, M. L., Jayaraman, M., Kazem, M., Maier, M. A., Srinivasulu, M., Weinstein, M. J., Chen, Q., Alvarez, R., Barros, S. A., De, S., Klimuk, S. K., Borland, T., Kosovrasti, V., Cantley, W. L., Tam, Y. K., Manoharan, M., Ciufolini, M. A., Tracy, M. A., de Fougerolles, A., MacLachlan, I., Cullis, P. R., Madden, T. D., Hope, M. J. Rational design of cationic lipids for siRNA delivery. *Nat Biotechnol* 28, 172-176 (2010).

Sharp, P. A. Split genes and RNA splicing. *Cell* 77, 805-815 (1994).

Sheng, M., Kim, M. J. Postsynaptic signaling and plasticity mechanisms. *Science* 298, 776-780 (2002).

Shiraishi, T., Nielsen, P.E. Photochemically enhanced cellular delivery of cell penetrating peptide-PNA conjugates. *FEBS Lett* 580, 1451-6 (2006).

Simeoni, F., Morris, M.C., Heitz, F., Divita, G. Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. *Nucleic Acids Res* 31, 2717-2724 (2003).

Slater, T. F., Sawyer, B., Straeuli, U. Studies on succinate-tetrazolium reductase systems. Iii. Points of coupling of four different tetrazolium salts. *Biochim Biophys Acta* 77, 383-393 (1963).

Somia, N., Verma, I. M. Gene therapy: trials and tribulations. *Nat Rev Genet* 1, 91-99 (2000).

Soomets, U., Lindgren, M., Gallet, X., Hällbrink, M., Elmquist, A., Balaspiri, L., Zorko, M., Pooga, M., Brasseur, R., Langel, Ü. Deletion analogues of transportan. *Biochim Biophys Acta* 1467, 165-176 (2001).

Spitzer, S., Eckstein, F. Inhibition of deoxyribonucleases by phosphorothioate groups in oligodeoxyribonucleotides. *Nucleic Acids Res* 16, 11691-704 (1988).

Srebrow, A., Kornblihtt, A. R. The connection between splicing and cancer. *J Cell Sci* 119, 2635-2641 (2006).

Stephenson, M. L., Zamecnik, P. C. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci U S A* 75, 285-288 (1978).

Sung, S. J., Min, S. H., Cho, K. Y., Lee, S., Min, Y. J., Yeom, Y. I., Park, J. K. Effect of polyethylene glycol on gene delivery of polyethyleneimine. *Biol Pharm Bull* 26, 492-500 (2003).

Swanson, J.A. Shaping cups into phagosomes and macropinosomes. *Nat Rev Mol Cell Biol* 9, 639-649 (2008).

Takei, K., McPherson, P. S., Schmid, S. L. and De Camilli, P. Tubular membrane invaginations coated by dynamin rings are induced by GTP-gamma S in nerve terminals. *Nature* 374, 186-190 (1995)

Takei, K., Haucke, V. Clathrin-mediated endocytosis: membrane factors pull the trigger. *Trends Cell Biol* 11, 385-391 (2001).

Thorén, P. E. G., Persson, D., Isaksson, P., Goksör, M., Önfeldt, A., Nordén, B. Uptake of analogs of penetratin, Tat(48-60) and oligoarginine in live cells. *Biochem Biophys Res Commun* 18, 100-107 (2003).

Tros de Ilarduya, C., Sun, Y., Düzgünes, N. Gene delivery by lipoplexes and polyplexes. *Eur J Pharm Sci* 40, 159-170 (2010).

Tong, A. W., Jay, C. M., Senzer, N., Maples, P. B., Nemunaitis, J. Systemic therapeutic gene delivery for cancer: crafting Paris' arrow. *Curr Gene Ther* 9, 45-60 (2009).

Vaishnaw, A. K., Gollob, J., Gamba-Vitalo, C., Hutabarat, R., Sah, D., Meyers, R., de Fougerolles, T., Maraganore, J. A status report on RNAi therapeutics. *Silence* 1, 14 (2010).

van den Berg, A., Mols, J., Han, J. RISC-target interaction: Cleavage and translational suppression. *Biochim Biophys Acta* 11, 668-677 (2008).

Venter, J.C. et al. The sequence of the human genome. *Science* 291, 1304-1351 (2001).

Viola, J. R., EL Andaloussi, S., Oprea, I. I., Smith, C. I. Non-viral nanovectors for gene delivery: factors that govern successful therapeutics. *Expert Opin Drug Deliv* 7, 721-35 (2010).

Vivés, E., Brodin, P., Lebleu, B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* 272, 16010-16017 (1997).

Wadia, J. S., Stan, R. V., Dowdy, S. F. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 10, 310-315 (2004).

Waehler, R., Russell, S. J., Curiel, D. T. Engineered targeted viral vectors for gene therapy. *Nat Rev Genet* 8, 573-587 (2007).

Wang, J., Lu, Z., Wientjes, M. G., Au, J. L. Delivery of siRNA therapeutics: barriers and carriers. *AAPS J* 12, 492-503 (2010).

Waters, J., Webb, A., Cunningham, D., Clarke, P. A., Raynaud, F., di Stefano, F., Cotter, F. E. Phase I clinical and pharmacokinetic study of Bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol* 18, 1812-1823 (2000).

Watson, J. D., Crick, F. H. C. A Structure for Deoxyribose Nucleic Acid. *Nature* 171, 737-738 (1953).

Watts, J. K., Deleavey, G. F., Damha, M. J. Chemically modified siRNA: tools and applications. *Drug Discov Today* 13, 842-855 (2008).

Wolff, J. A., Williams, P., Acsadi, G., Jiao, S., Jani, A., Chong, W. Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques* 11, 474-485 (1991).

Wolf, Y., Pritz, S., Abes, S., Bienert, M., Lebleu, B., Oehlke, J. Structural requirements for cellular uptake and antisense activity of peptide nucleic acids conjugated with various peptides. Biochemistry 45, 14944-14954 (2006).

Wolfert, M. A., Schacht, E. H., Toncheva, V., Ulbrich, K., Nazarova, O., Seymour, L. W. Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. *Hum Gene Ther* 7, 2123-2133 (1996).

Xu, L., Anchordoquy, T. Drug delivery trends in clinical trials and translational medicine: challenges and opportunities in the delivery of nucleic acid-based therapeutics. *J Pharm Sci* 100, 38-52 (2011).

Yacyshyn, B. R., Bowen-Yacyshyn, M. B., Jewell, L., Tami, J. A., Bennett, C. F., Kisner, D. L., Shanahan, W. R. A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease. *Gastroenterology* 114, 1133-1142 (1998).

Yin, H., Moulton, H. M., Seow, Y., Boyd, C., Boutilier, J., Iverson, P., Wood, M. J. A. Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function. *Hum Mol Genet* 24, 3909-3918 (2008).

Yin, H., Saleh, A. F., Betts, C., Camelliti, P., Seow, Y., Ashraf, S., Arzumanov, A., Hammond, S., Merritt, T., Gait, M. J., Wood, M. J. Pip5 transduction peptides direct high efficiency oligonucleotide-mediated dystrophin exon skipping in heart and phenotypic correction in mdx mice. *Mol Ther* 19, 1295-1303 (2011).

Zamecnik, P. C, Stephenson, M. L. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci U S A* 75, 280-284 (1978).

Zhang, G., Wooddell, C. I., Hegge, J. O., Griffin, J. B., Huss, T., Braun, S., Wolff, J. A. Functional efficacy of dystrophin expression from plasmids delivered to mdx mice by hydrodynamic limb vein injection. *Hum Gene Ther* 21, 221-37 (2010).

Zhou, X. H., Klibanov, A. L., Huang, L. Lipophilic polylysines mediate efficient DNA transfection in mammalian cells. *Biochim Biophys Acta* 1065, 8 -14 (1991).

Zimmermann, T. S, Lee, A. C., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M. N., Harborth, J., Heyes, J. A., Jeffs, L. B., John, M., Judge, A. D., Lam, K., McClintock, K., Nechev, L. V., Palmer, L. R., Racie, T., Rohl, I., Seiffert, S., Shanmugam, S., Sood, V., Soutschek, J., Toudjarska, I., Wheat, A. J., Yaworski, E., Zedalis, W., Koteliansky, V., Manoharan, M., Vornlocher, H. P., MacLachlan, I. RNAi-mediated gene silencing in non-human primates. *Nature* 441, 111-4 (2006).

Zorko, M., Langel, Ü. Cell-penetrating peptides: mechanisms and kinetics of cargo delivery. *Adv Drug Deliv Rev* 57, 529-545 (2005).