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Delivery of nucleic acids by cell-penetrating peptides: application in modulation of gene expression

TARTU UNIVERSITY PRESS

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Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on April 29th, 2011 by the council of the Institute of Technology, Faculty of Science and Technology, University of Tartu.

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Commencement: Auditorium 121, Nooruse 1, Tartu, at 14.15 on June 17th, 2011

Publication of this dissertation is granted by the University of Tartu

ISSN 2228–0855
ISBN 978–9949–19–676–0 (trükis)
ISBN 978–9949–19–677–7 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 291

ABSTRACT

Scientific advances over the last couple of decades have witnessed the emergence of a wide variety of methods to manipulate gene expression. Nucleic acids and their analogues, ranging from shorter oligonucleotides to full-size plasmids, form the battery of molecules that can be utilized for gene expression modulation. Unfortunately, the physicochemical properties of these molecules impede their translocation across biological membranes, thus, in order to reach their active sites within cells, they require assistance in their intracellular transport.

Cell-penetrating peptides (CPPs) are one class of non-viral delivery vectors, which have been shown to facilitate the delivery of various types of bioactive cargos, including plasmid DNA (pDNA), splice-correcting oligonucleotides (SCOs) and small interfering RNAs (siRNAs), both *in vitro* and *in vivo*.

This thesis aims for the development and characterization of novel CPP-based vectors with improved delivery properties for nucleic acid-based molecules. That includes the delivery of pDNA for gene transfer, SCOs for splicing correction and siRNAs for gene silencing, by non-covalent nanoparticle formation approach, both *in vitro* and *in vivo*. We modify our CPPs by adding stearic acid and/or a novel endosomotropic modification or by making amino acid substitutions in the peptide backbone. We show that stearic acid modification of the pre-clinically used $(RxR)_4$ peptide allows efficient delivery of pDNA and SCOs *in vitro*. Moreover, addition of stearic acid to another amphipathic CPP, TP10, generates a vector that, not only, enables efficient pDNA delivery *in vitro*, but also *in vivo*, following local delivery. Next, we describe the introduction of a novel endosomotropic modification to stearylated TP10 peptide, creating an extremely efficient vector for the delivery of siRNAs, namely PepFect6. PepFect6/siRNA nanoparticles mediate ubiquitous gene silencing in a battery of refractory cells and also promote significant gene silencing upon systemic delivery *in vivo*. Finally, we show how replacement of lysines with ornithines in the strearyl-TP10 backbone creates an exceptionally efficient vector for the delivery of SCOs, which induces significant splice correction at low doses in a disease-relevant *in vitro* Duchenne muscular dystrophy model. Conclusively, these novel vectors show remarkable delivery potential for the delivery of gene modulating compounds both *in vitro* and *in vivo*.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the thesis by Roman numerals:

I. **Lehto, T,** Abes, R, Oskolkov, N, Suhorutšenko, J, Copolovici, DM, Mäger, I, Viola, JR, Simonson, OE, Ezzat, K, Guterstam, P, Eriste, E, Smith, CIE, Lebleu, B, EL Andaloussi, S, and Langel, Ü (2010). Delivery of nucleic acids with a stearylated $(RxR)_4$ peptide using a non-covalent co-incubation strategy. *J Control Release.* **141**: 42–51.

II. **Lehto, T,** Simonson, OE, Mäger, I, Ezzat, K, Sork, H, Copolovici, DM, Viola, JR, Zaghloul, EM, Lundin, P, Moreno, PMD, Mäe, M, Oskolkov, N, Suhorutšenko, J, Smith, CIE, and EL Andaloussi, S (2011). A peptide-based vector for efficient gene transfer *in vitro* and *in vivo*. *Mol Ther.* In press.

III. EL Andaloussi, S, **Lehto, T,** Mäger, I, Rosenthal-Aizman, K, Oprea, II, Simonson, OE, Sork, H, Ezzat, K, Copolovici, DM, Kurrikoff, K, Viola, JR, Zaghloul, EM, Sillard, R, Johansson, HJ, Hassane, FS, Guterstam, P, Suhorutšenko, J, Moreno, PMD, Oskolkov, N, Hälldin, J, Tedebark, U, Metspalu, A, Lebleu, B, Lehtiö, J, Smith, CIE, and Langel, Ü (2011). Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically *in vivo*. *Nucleic Acids Res*. In press.

IV. Ezzat, K, EL Andaloussi, S, Zaghloul, EM, **Lehto, T,** Lindberg, S, Magdy, T, Viola, JR, Guterstam, P, Moreno, PMD, Abdo, R, Sillard, R, Hammond, SM, Wood, MJA, Arzumanov, AA, Gait, MJ, Smith, CIE, Hällbrink, M, and Langel, Ü (2011). PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. *Nucleic Acids Res*. In press.

The articles listed above have been printed with the permission of the copyright owners. My personal contribution of the articles referred to in this thesis is as follows:

I. designed and performed most of the experiments, analyzed all the data, wrote the paper as a corresponding author;

II. designed and performed most of the experiments, analyzed all the data, wrote most of the paper as a corresponding author;

III. designed and performed many of the experiments, analyzed most of the data and participated in the writing of the paper;

IV. performed some of the experiments and participated in the writing of the paper.

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ABBREVIATIONS

2′-OMe	2′-O-methyl
ALT	alanine aminotransferase
asON	antisense oligonucleotide
AST	aspartate aminotransferase
BMD	Becker muscular dystrophy
CPP	cell-penetrating peptide
CR	charge ratio
CRP	C-reactive protein
DLS	dynamic light scattering
DMD	Duchenne muscular dystrophy
DRBD	double-stranded RNA-binding domain
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FACS	fluorescence-acivated cell sorter
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
HPRT1	hypoxanthine phosphoribosyltransferase 1
LF2000	Lipofectamine 2000
LNA	locked nucleic acid
LNP	lipid nanoparticle
Luc	luciferase
MAP	model amphipathic peptide
MEND	multifunctional envelope-type nanodevice
MR	molar ratio
MSP	muscle specific heptapeptide
NLS	nuclear localization signal
ON	oligonucleotide
pDNA	plasmid DNA
PEG	polyethyleneglycol
PEI	polyethyleneiminine
PF3	PepFect3
PF5	PepFect5
PF6	PepFect6
PF14	PepFect14
Pip	PNA internalization peptide
PMO	phosporodiamidate morpholino oligonucleotide
PNA	peptide nucleic acid
POD	peptide for ocular delivery
RFP	red fluorescent protein
RISC	RNA-induced silencing complex

RNAi	RNA interference
RT-PCR	reverse-transcription polymerase chain reaction
RT-qPCR	quantitative reverse-transcription polymerase chain reaction
RVG	rabies virus glycoprotein
SCO	splice-correcting oligonucleotide
siRNA	small interfering RNA
TP10	transportan 10
VEGF	vascular endothelial growth factor

INTRODUCTION

In light of the scientific progress over the last couple of decades a wide variety of approaches have emerged that can be used to access gene expression and that has opened a new avenue for treating the diseases that were considered untreatable with conventional methods. Moreover, many of these potential therapy platforms are slowly but firmly moving towards the pre-clinical and clinical stages. Generally, molecules utilized for intervening with the expression of genes are based on nucleic acids and their analogues, ranging from shorter DNA/RNA oligomers to full-length plasmids, and to all of these molecules it will be referred to as oligonucleotides (ONs) hereafter. Unfortunately, the physicochemical properties impede the translocation of ONs over cellular membranes and therefore ONs require the assistance of efficient delivery vectors in order to reach their intracellular targets. Viral vectors have been shown to be very efficient, but their applicability is hampered by the lack of safety and they are also not compatible with the delivery of short ON analogues. This has led to significant interest in non-viral delivery vectors.

Cell-penetrating peptides (CPPs) are one class of such non-viral delivery vectors that, since the initial discovery in 1994 [1], have been successfully used for the intracellular delivery of various bioactive cargos, including plasmid DNA (pDNA), splice-correcting ONs (SCOs) and small interfering RNAs (siRNAs), both *in vitro* and *in vivo*. For this, CPPs are under intense investigations and by today many of the CPP-based platforms have reached the pre-clinical studies.

This thesis is dedicated to the development of CPP-based vectors with improved delivery properties. The CPPs used in this thesis are designed to enable the direct non-covalent nanoparticle formation with different ON-based cargos, including pDNA for gene delivery, SCOs to modulate splicing, and siRNAs to induce gene silencing. All these vectors are thoroughly studied for the *in vitro* delivery of the mentioned cargos, while some of them are further tested for their applicability for *in vivo* delivery.

1. LITERATURE OVERVIEW

1.1 Regulation of gene expression with ONs

There is a variety of methods available to modulate gene expression. For instance, deficient gene expression can be restored by expressing it from an exogenously introduced genetic material. Furthermore, gene expression can be targeted by the antisense-based approaches, for example to silence genes by RNA interference (RNAi) or to enable the production of a functional protein by manipulating splicing patterns. Additionally, there are other possibilities how to regulate gene expression, such as using the decoy ONs to sequester transcription factors, microRNAs to suppress the gene expression by endogenous RNAi machinery, anti-microRNAs (antiMiRs) to target naturally occurring microRNAs and enhance their target gene expression, to name a few. These antisense techniques are unique because: 1) they are sequence specific and affect only individual genes, properties which are lacking in conventional medicine; 2) all genes are amenable for antisense targeting; 3) ONs are very easy to design as only the primary RNA/DNA target sequence is needed and; 4) ONs can target proteins that are undruggable with conventional small molecule drugs. While the mechanisms behind these effects are different, there is a common denominator that ties all of these processes together – namely, that the effector molecules are mostly based on ONs and their analogues. Below there is a more detailed description of the gene expression modulation methods utilized in this thesis (also, see Figure 1).

1.1.1. Gene therapy

Gene therapy is an approach that aims to cure diseases that are associated with the loss of gene function by the addition/substitution of genes and thereby correcting the normal gene function [2]. Advances of the biomedical science and the completion of the Human Genome Project [3, 4] have led to the identification of a wide variety of mutated genes that cause diseases. Originally gene therapy was developed to targeted hereditary monogenic disorders, for example haemophilia [5], muscular dystrophies [6] and cystic fibrosis [7], however, recently the range of treatable diseases has been expanded and the applicability of gene therapy can be easily envisioned for cardiovascular [8, 9], neurological diseases [10], cancer [11, 12], and others.

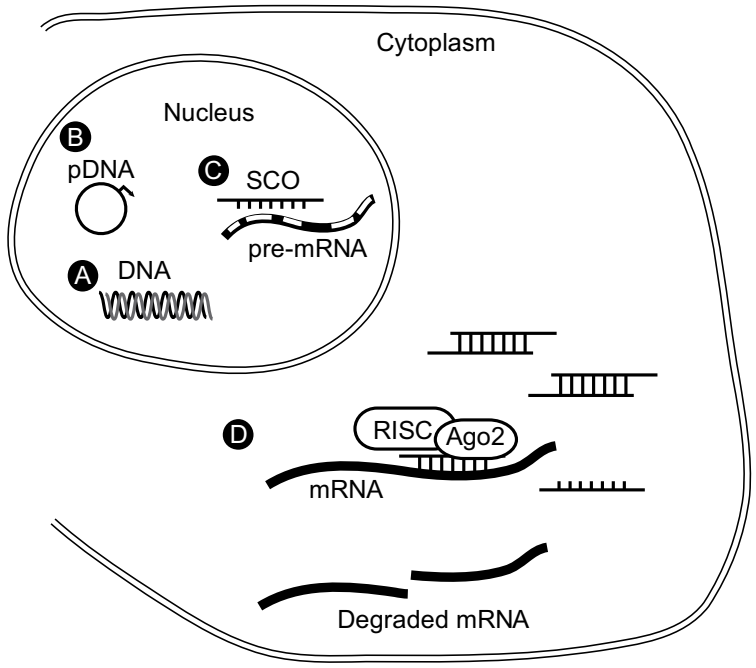

Figure 1. A schematic overview of gene therapy and gene expression modulation approaches. A) Gene is expressed from a viral vector in the nucleus. B) Gene is expressed from plasmid DNA (pDNA) in the nucleus. C) Splice-correcting oligonucleotides (SCOs) bind to pre-mRNA and modulate the splicing pattern in the nucleolus. D) RNAi induction after the siRNA is incorporated into the RISC complex and degrades complementary mRNA in the cytoplasm.

A fundamental requirement of gene therapy is to deliver a gene with its regulatory elements to the nucleus of cells with high efficiency and further be expressed there. Viral vectors are vigorously used for the gene delivery purpose. Recombinant viruses, like retroviruses, lentiviruses, adenoviruses, adeno-associated viruses and herpes simplex viruses, have been utilized for the incorporation of genes of interest into their genome, and have shown to be extremely efficient in delivering of genetic material into cell nucleus [13, 14]. Consequently, viral vectors have been utilized in a variety of clinical trials. For example, in 2000, Cavazzano-Calvo et al. reported a successful clinical trial for children with severe combined immunodeficiency (SCID)-X1 [15] and Kay et al. with the factor XI in haemophilia clinical trial [16].

Different diseases that can be targeted with gene therapy require distinct duration and levels of gene expression. For instance, high levels of transient expression over a short-term period could be beneficial for cancer treatment, whereas low levels of long-term expression after the integration into genome might be necessary for hereditary genetic disorders [13]. While there are viral

vectors that enable both transient and stable expression, both of these methods are potentially associated with several side-effects. General adverse effects include acute toxicity arising from the infusion of foreign material, immune responses targeted towards the transduced cells, and humoral immune response against the endogenously introduced gene and/or its products. Moreover, there are additional hazard with integrating vectors, as it is known that the genome integration of viral vectors can activate oncogenes and induce leukaemias [13].

Even though these drawbacks have curbed the initial optimism and caused significant concerns over the overall applicability of gene therapy [17] it has evoked even more intense investigations how to develop more effective and safer viral vectors, while, importantly, it has also turned the attention to the non-viral alternatives.

1.1.2. Antisense technology

Antisense technology holds an immense potential by theoretically enabling regulation of expression of every single gene. It takes advantage of the 15–25 base long oligonucleotides that have been designed to be complementary to specific genes or mRNAs and to affect the gene expression at transcriptional or post-transcriptional levels, respectively [18, 19]. Hence, antisense oligonucleotides (asONs) can act by inhibiting transcription from chromosomal DNA [20, 21] or causing translational arrest of the mRNA, most commonly either by recruiting RNase H for the degradation of target sequence or by sterical hindrance of the ribosomal assembly on mRNA [22–24]. The first report showing that gene expression could be modified by the introduction of exogenous nucleic acids came from Patterson and colleagues, who used single-stranded DNA to inhibit the translation of complementary RNA in cell-free system [25]. Short after, in 1978, Zamecnik and Stephenson showed, what is now considered as a proof-of-principle study for antisense technology, that a short DNA oligonucleotide antisense to the Rous sarcoma virus could inhibit viral replication *in vitro* [26, 27].

1.1.3. Splice correction

The human genome project revealed that the number of human genes was previously greatly overestimated and that only around 20500 genes are responsible for the significantly larger repertoire of proteins [3, 4]. Alternative splicing is a key post-transcriptional mechanism that takes place in the nucleolus of cells and is carried out by the splicing machinery called spliceosome. In case of constitutional splicing the immature pre-mRNA transcript is always processed in the same way and exons (coding sequences) are joined together as they occur in the transcript. However, different mRNA transcripts from the same gene can be generated through alternative splicing, for example through exon skipping, and

this process is one of the main contributors to the protein diversity. Alternative splicing is driven by the so-called 5′ and 3′ splice sites (invariant GU and AG intronic nucleotides), a branch site, polypyrimidine tract and other regulatory regions that are recognized by spliceosome which catalyzes the accurate cleavage and the rejoining of exons [28, 29]. Disruption of the normal splicing patterns by disease-causing mutations can lead to the manifestation of a variety of diseases, such as muscular dystrophies, cystic fibrosis, β-thalassaemia, cancer and several neurological disorders [30–32]. The majority of diseases associated with aberrant splicing are caused by the single nucleotide substitutions in intronic or exonic segments of the splicing sites and these lead to either exon skipping, utilization of pseudo splicing sites or retention of the mutated intron. Moreover, mutation can introduce a new splice site within the intron or exon.

SCOs are utilized to modulate pre-mRNA splicing by binding to the pre-mRNA and blocking the access to the transcript for the spliceosome and other splicing factors. The effects of SCOs can lead to restoration of correct splicing, production of novel splice variant or directing splicing from one splice variant to another [33]. Early work of the group of Ryszard Kole demonstrated that SCOs could be used to correct the aberrantly spliced pre-mRNA of human β-globin in a disease model of β-thalassemia [34–36]. Since then, SCOs have been widely utilized to target aberrant splicing in many disease models and the technology has lately been exploited in clinical trials [37].

For instance, mutations in the dystrophin gene can lead to Duchenne muscular dystrophy (DMD), which is a severe disease with progressive degenerative myopathy, or to its milder allelic disorder, Becker muscular dystrophy (BMD) [38]. Most nonsense mutations within this gene cause shift in the translational reading frame and termination of the protein synthesis, therefore, complete absence of the functional dystrophin, manifesting in DMD. In the case of BMD, nonsense mutations occur in the regulatory sequence and cause the partial in-frame skipping of an exon and the production of partially functional dystrophin protein. SCOs have been successfully used to convert the DMD phenotype of a disease to BMD by removing one or more of the exons (exon skipping), resulting in the production of partially functional dystrophin. SCOs targeting DMD are in clinical trials in Europe, as reviewed in [39–42]. In case of exon skipping, SCOs are also referred to as splice-switching ONs (SSOs).

Regular asONs usually are designed to degrade the complementary mRNA and this is mediated by recruiting RNase H enzyme that facilitates the degradation of the target sequence. Contrary to the latter, asONs used for splicing correction (SCOs) need to be resistant to the RNase H as the goal of this strategy is to only manipulate the splicing of the transcript without degrading it. Therefore in order to prevent RNase H incorporation, different ON chemistries have been introduced, for example locked nucleic acids (LNAs) [43], 2′-O-methyl (2′-OMe) [44] and 2′-O-methoxyethyl (MOE) [45] ribose modifications or phosporodiamidate morpholinos (PMOs) [46] and peptide nucleic acids (PNAs) [47]. In many cases these different RNA analogues have greater affinity

towards the complementary RNA and are, importantly, more resistant to nuclease digestion as compared to the regular RNA.

1.1.4. RNA interference

RNAi is an integral pathway in eukaryotic cells by which sequence-specific siRNA can target and induce cleaving of complementary RNA. RNAi is activated by the longer double-stranded RNA fragments, which are processed into small interfering RNA (siRNAs) by the Dicer enzyme [48]. These 21–23 nucleotide long double-stranded siRNAs are thereafter incorporated into a multiprotein complex termed RNA-induced silencing complex (RISC) [49, 50]. In the RISC complex, siRNA is unwound by the catalytic Argonaute 2 protein and the sense strand (passenger strand) is cleaved from siRNA [51]. Active RISC, comprising the antisense strand (guide strand) of the siRNA selectively binds to complementary mRNA and degrades it. Interestingly, activated RISC can additionally associate with another target mRNA and thus leading to propagation of and prolonged gene silencing [52]. As a result, therapeutically relevant gene silencing from 3–7 days can be achieved in rapidly dividing cells and even more in non-dividing cells [53].

RNAi moved into the spotlight in 1998 when Fire, Mello and colleagues discovered that introduction of a double-stranded RNA induced gene expression silencing in *Caenorhabidis elegance (C. elegance*) [54]. In 2001, Elbashir et al. published proof-of-principle study, which showed that RNAi can be induced also after the introduction of synthetic siRNA to cells, which produced sequence-specific gene silencing *in vitro* [55]. This was soon followed by the first successful report on efficient gene silencing *in vivo*, targeting hepatitis C [56]. Since then, the RNAi field has blossomed and siRNA-based therapeutics have now reached into clinical trials, as reviewed in [57–61].

1.2. Vectors for the delivery of ON-based molecules

As mentioned above, compounds used in the gene therapy are based on ONs and their analogues. Unfortunately, these molecules are usually charged and have high molecular weight, which makes them essentially impermeable over cellular membranes without assistance of a delivery vector. Broadly, vectorization of ONs can be divided into two categories – viral and non-viral delivery vectors. As aforementioned, viral vectors are very efficient, however safety concerns have severely hampered their utilization in clinics recently. Moreover, viral vectors are restricted by cargo-carrying capacity and are not compatible with the transient delivery of shorter antisense-based ONs, for instance SCOs and siRNAs. This has changed the focus of the field significantly towards the

non-viral delivery, to find safer alternatives for gene delivery and creating vectors for shorter ONs. Different delivery approaches for ONs are presented in Figure 2.

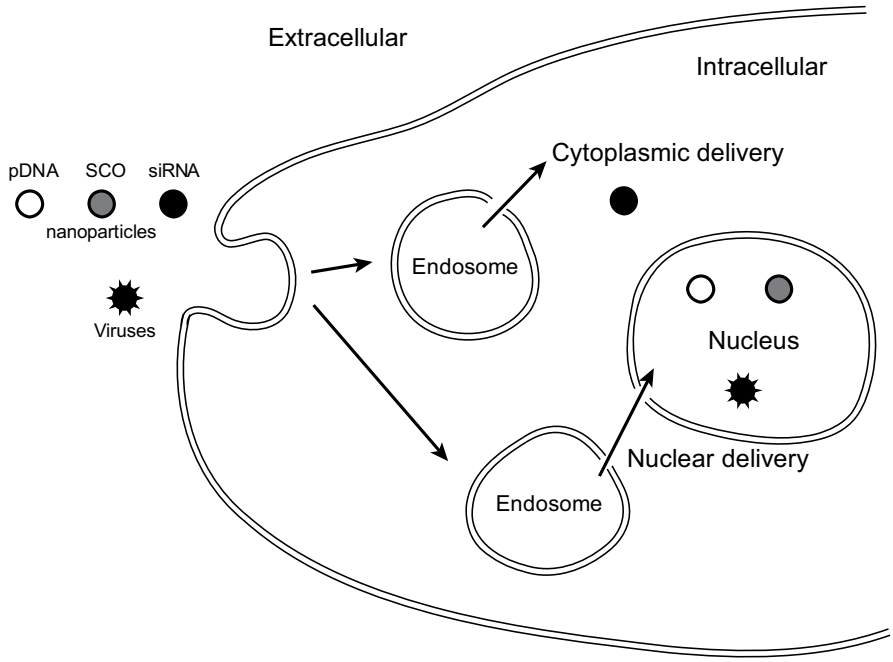

Figure 2. Overview of the different delivery methods and their intracellular targets. Non-viral delivery vectors usually form nanoparticles with ON-based cargo and these particles are endocytosed and subsequently sequestered in the endosomes. For the cargo molecules to reach their active sites these particles need to escape from the endosomes. If siRNA is the cargo molecule then it requires rapid endosomal escape in order to reach the cytoplasm, where the RNAi machinery resides, while for SCOs and pDNA, it is presumably more advantageous if they would escape later in the endo/lysosomal pathway to reach to the nucleus. Viral vectors are naturally programmed to reach to the nucleus, usually by endosomal pathways.

1.2.1. Non-viral delivery

Non-viral delivery vectors are usually based on different kind of peptides, lipids or polymers that bind ONs and their analogues by electrostatic/hydrophobic interactions and facilitate their condensation into nano-sized particles that mediate their intracellular delivery. Non-viral delivery strategies have been recently reviewed in a variety publications, for pDNA [62–65], SCOs [33, 39, 66] and siRNAs [58–61].

The two most widely utilized platforms in non-viral delivery are based on cationic lipids or synthetic polymers, which are known under the name of

lipoplexes and polyplexes, respectively. The delivery potential of cationic lipids were first reported in 1987 [67], when it was shown that they are capable of binding pDNA and facilitating its intracellular delivery. Since then many transfection methods based on cationic lipids have emerged. Cationic lipids are most frequently exploited vectors for pDNA delivery and many initial gene therapy clinical trials have also used this technology. Moreover, this platform has also been successfully applied for the delivery of shorter ONs, for instance SCO and siRNAs. Unfortunately, lipofection has been shown to be associated with the acute toxicity *in vitro* and *in vivo* [68, 69]. Moreover, it has been reported that it is complicated to reproduce the cationic lipid-based formulations and instability *in vivo* is another obstacle that hamper their use [63].

The other most used platform, polyplexes, is based on different cationic polymers acting as ON-binding motifs and delivery vehicles. A wide variety of different chemistries form the basis of these polymers and these materials could be originating from simple "off-the-shelf" products, such as polylysines, polyethyleneimines (PEIs), polyamidoamines (PAMAM dendrimers), to specially designed delivery vectors, for instance imidazole-containing, membrane-disruptive and cyclodextrin-containing polymers [60, 63]. As with cationic lipids, these polymers have been intensely studied for the delivery of pDNA, but also for shorter ONs.

Extensive studies with a variety of non-viral vectors have increased the understanding of what is required from an ideal delivery vector. To design delivery vehicles the following aspects have to be considered. First of all there are a variety of physicochemical properties, like binding and condensing of the ONs with suitable size, shape, surface charge and stability in solution. Secondly, there are general *in vitro* characteristics, such as stability in the transfection media, including the presence of serum, association with membrane and cellular internalization, endosomal escape, cytoplasmic trafficking, nuclear internalization, if necessary, dissociation of the nanoparticles before or at the site of action of the cargo, either in the cytoplasm or nucleus. Thirdly, if these particles are a subject to the *in vivo* delivery then there are additional barriers, such as stability and survival in the blood stream, penetration of the blood vessel wall and surrounding tissue and ideally binding to the specific cells and of course subsequent intracellular delivery. These aspects have been lately extensively analyzed in many excellent reviews [60, 63, 65]. Generally, a wide variety of these required properties is still complicated to control, especially simultaneously, and creation of nanoparticles with ideal properties is something beyond scientific knowledge and capacity at the moment.

1.3. Cell-penetrating peptides (CPPs)

Peptides and proteins were long considered incapable of crossing cellular membranes, as they have large molecular weight and are often hydrophilic. This dogma was changed in 1988, when two different groups simultaneously

reported that both a recombinant and synthetic 86 amino acid long Tat protein could translocate over cellular membranes in cell cultures [70, 71]. A couple of years later it was reported by the group of Alain Prochiantz that, similarly to the Tat protein, the homeodomain of Antennapedia (a *Drosophila* homeodomain) was capable of cellular internalization [72], followed by the discovery in 1994 that only the 16-mer peptide derived from its third helix is required and sufficient to confer intracellular delivery [1]. This peptide was named penetratin (also termed pAntp) and this landmark finding marked the birth of the field of CPPs. Since then many CPPs has been discovered with different origin and by today the number of different CPPs that has been reported is above 100.

By definition, CPP *are relatively short peptides, 5–40 amino acids in length, with the ability to gain access to the cell interior by means of different mechanisms, mainly including endocytosis, and with the capacity to promote the intracellular delivery of covalently or noncovalently conjugated bioactive cargos* [73]. While CPP are hard to define, the common denominator between all the CPP is that they are capable of mediating the delivery of various macromolecules across the cellular membranes, including peptides, proteins, pDNA, asONs, SCOs, siRNAs, antiMiRs, and nanoparticles both *in vitro* and *in vivo*, as reviewed in [74–80].

CPPs can be classified by different means and the most recognized classification system is based on their origin [73]. By this, CPPs are divided into three: 1) protein derived – comprising classical CPPs that have been derived from naturally occurring proteins, for example penetratin [1] and Tat [81]; 2) chimeric – gathering CPPs that at least partially share sequences from naturally occurring proteins, e.g. transportan [82] and MPG [83]; and 3) synthetic – CPPs with solely designed sequences with translocating properties, e.g. model amphipathic peptide (MAP) [84] and polyarginines [85]. While this system gives an indication of how particular CPPs have been found or designed, it does not take into account the physicochemical properties of these peptides. Some examples of the CPPs are presented in the Table 1.

Another classification has been proposed lately by André Ziegler [86], a view that is also supported by many others [87–89], which is based on the individual properties of CPPs upon the association with lipid membranes. According to this classification system, CPPs are also divided into three categories: 1) primary amphipathic; 2) secondary amphipathic; and 3) non-amphipathic peptides.

Primary amphipathic peptides comprise both hydrophobic and cationic domains in their primary sequence and they are known to bind both neutral and anionic lipids with high affinity, which suggests that their membrane activity is mostly dependent on the hydrophobic interactions [90–92]. As these CPPs act very similarly to surface active agents, for instance decreasing the surface tension, they are known to penetrate deep into to the lipid membranes [91]. As a result, theories of direct penetration are based on the way primary amphipathic

CPPs interact with lipid membranes [93]. Many efficient CPPs belong to this group, for instance MPG [83], transportan [82], TP10 [94] and Pep-1 [95].

Secondary amphipathic peptides have generally been shown to have poor affinity towards neutral membranes [92, 96]. Similarly to primary amphipathic CPPs, these peptides also contain hydrophobic and hydrophilic domains, however, they become amphipathic by adopting secondary structures like α-helices [85, 97] and β-sheets [98] upon interaction with lipid membranes. It seems in general that their membrane activity correlates with high composition of anionic lipids in the membrane. Penetratin is by far the most studied CPP in this group, however, many designed CPPs fall into this group, for example MAP [84] and CADY [99].

The third group contains non-amphipathic peptides which do no associate with lipid membranes unless they contain high content of monovalent anionic lipids [92]. Usually these peptides do not perturb membranes [100, 101]. Many cationic CPPs fall into this category, for instance Tat [81] and polyarginines [85]. Interestingly, if cationic peptides have been modified to be amphipathic [102, 103], for example by acylation [104], their activity and toxicity towards the membranes is also greatly enhanced.

Table 1. Examples of different CPPs

CPP	Sequence	Origin	Ref.
Primary amphipathic			
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2	Chimeric	[82]
TP10	AGYLLGKINLKALAALAKKIL-NH_2	Chimeric	[94]
MPG	GALFLGWLGAAGSTMGAPKKKRKV-cya	Chimeric	[83]
Pep-1	KETWWETWWTEWSQPKKKRKV-cya	Chimeric	[95]
Secondary amphipathic			
Penetratin	RQIKIWFQNRRMKWKK-COOH	Protein derived	[1]
MAP	KLALKLALKALKAALKLA-NH_2	Synthetic	[84]
CADY	GLWRALWRLLRSLWRLLWRA-cya	Synthetic	[99]
Non-amphipathic			
Tat (48–60)	GRKKRRQRRRPPQ-COOH	Protein derived	[81]
Oligoarginine	$(R)_n$	Synthetic	[85]

Cya, cysteamide.

1.3.1. Internalization mechanisms

Broadly there are two ways that CPPs are believed to gain access to the cell interior – by endocytic pathways or direct penetration. Earlier uptake studies were mostly conducted with fluorophore-labeled peptides by visualizing the uptake by fluorescence microscopy or by flow cytometry (FACS) without assigning biological activity. These studies showed that the uptake of CPPs was

not inhibited by energy depletion, low temperature or endocytosis inhibitors. For many CPPs, their all-D-analogues were internalized with similar efficiency, indicating that selective cell surface receptors were not involved in the uptake mechanism. Based on this information it was believed that CPPs internalize by direct, physical transfer through the cellular membranes [1, 81, 105]. This understanding was revised when it became evident that cell fixation could cause artifactual redistribution of the peptides [106, 107]. This has caused the re-evaluation of the uptake mechanism of CPPs and now it is widely accepted that CPPs exploit an energy-dependent internalization mechanism that involves binding to cellular membrane heparan sulfates [108, 109] and endocytosis of different types, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and clathrin- and caveolae-independent endocytosis, as extensively described in [74, 76, 80, 110, 111]. Nevertheless, direct penetration pathway cannot be excluded, as it is still often reported that depending on the particular CPP, cargoes and delivery conditions, it might be responsible for translocation. For example, MPG and CADY peptide have been shown to internalize by non-endocytic internalization mechanism [112]. Overview of the uptake pathways of CPPs can be seen in Figure 3.

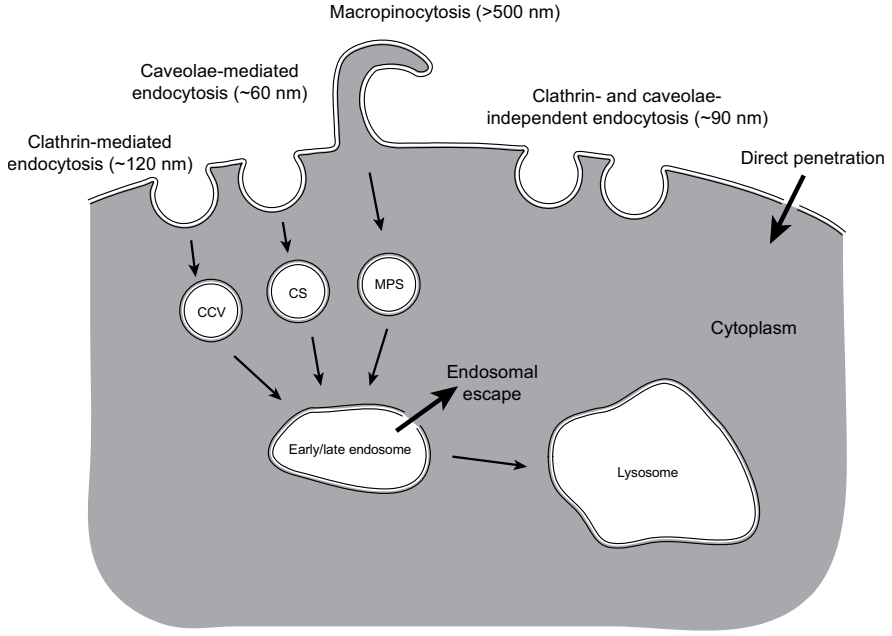

Figure 3. Overview of the different uptake pathways of CPPs. CPPs are known to be internalized into cells by two pathways – either by different endocytic pathways or by direct translocation mechanism. Endocytosis comprizes different pathways including clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and clathrin- and caveolae-independent endocytosis. If CPP and associated cargo is internalized by endocytosis it ends up in the endosomes and for the cargo to reach their active sites they need to escape endocytic compartments or they will undergo lysosomal degradation. CCV, clathrin-coated vesicle; CS, caveosome; MPS, macropinosome.

Even though it is accepted that CPPs gain access to the cells by endocytosis, especially when associated with cargo, it is not clear what exact pathways do different CPP utilize. For example, Tat peptide has been shown to internalize by clathrin-mediated endocytosis in some reports [113, 114], while others have also indicated the involvement of caveolae-mediated endocytosis [115] and micropinocytosis [116]. There is also evidence that CPPs can utilize different endocytic pathways in parallel [111, 117, 118], whereas a higher CPP concentration might also trigger direct translocation mechanism [117, 119]. This uncertainty can also be dependent on the fact that endocytosis inhibitors that are mostly used to elucidate the uptake pathways are not specific enough or have been used at inappropriate concentrations [120]. In addition, it has also been shown that inhibition of one pathway might lead to transport through others [121]. Conclusively it seems that choice of internalization pathway is dependent on the particular CPP, utilized concentrations, cargo, cargo coupling methods, cell lines, experimental conditions, to name a few.

1.3.2. Endosomal entrapment and escape

When using endocytic pathways for cellular internalization, CPPs and their respective cargo get sequestered in endosomal compartments and are not available at their active sites that usually reside outside of the endosomes. Thus, endosomal entrapment serves as a main limiting step in the bioavailability of CPPs (also see Figure 3). This has led to intense investigations to overcome this limitation, from design of peptides with amphipathic properties that are more active towards the endosomal membranes to the more specific modifications that would add endosomotropic properties, like membrane-disruptive peptides, polymers, fusogenic lipids (as recently reviewed in [122–125]).

For instance, fusogenic HA2 peptide derived from the HA2 protein of an influenza virus is known to enable endosomal escape by adopting α-helical structure at endosomal pH and this mediates partial disruption of the endosomal membrane. Wadia et al. were first to utilize this peptide with CPPs and they showed that by adding this segment to the Tat peptide it enhanced the uptake of Tat-Cre fusion protein [116]. Histidines-rich motifs have been used to augment the endosomotropic properties. For example, Lundberg et al. designed a penetratin analogue by introducing histidine moieties to induce the formation of α-helical structure in the early/late endosomes and promote endosomal escape [126]. Lo et al. used similar approach and showed that incorporation of histidines into Tat peptide significantly enhanced the pDNA delivery both *in vitro* and *in vivo* [127]. Histidines are believed to enable endosomal escape by being protonated at low pH, leading to the subsequent swelling and rupture of the endosomes. Fusogenic lipids have been frequently added to the CPP formulations to enhance the endosomal escape. For example, dioleoylphosphatidylethanolamine (DOPE) has been used as an integral part of the multifunctional

envelope-type nanodevice (MEND) platform that has been shown to promote endosomal escape [128, 129].

Lately, the addition of fatty acid modification to CPPs has been shown to enhance the bioavailability of the associated cargo. Particularly, addition of stearic acid modification to CPPs has been shown to increase the delivery efficiency of pDNA [85, 130], SCOs [131] and siRNAs [132, 133]. Examples of the stearic acid-based modification on CPP and their utilization for the delivery of ONs will be more thoroughly described in the next sections.

1.4. Vectorization of ONs with CPPs

In general there are two main strategies how CPPs can vectorize cargo – covalent and non-covalent method. Most of the studies in the CPPs field have utilized covalent conjugation method for cargo attachment. It is understandable why covalent conjugation has been preferred, as the chemical conjugation results in a well-defined chemical entity and, if looking on the clinical applicability point of view, this is desired. However, as every methodology, this strategy also has its drawbacks, for example: it is very cumbersome to generate conjugates with charged ONs; it is not compatible with the delivery of large nucleic acids, like plasmids; it seems that covalent conjugation is also not compatible with siRNA delivery; and this strategy is very laborious and costly. Based on this, non-covalent strategy has gained momentum lately.

Non-covalent strategy for vectorization of ONs with CPPs was first introduced in 1997 by the group of Heitz and Divita with the MPG peptide [134]. Since then this approach has been extended to a variety of bioactive cargos, for example pDNA, peptides, proteins, siRNAs, SCOs. In case of ONs and their analogues, this strategy relies on electrostatic interaction, where positive charge of the CPPs enable the neutralization of the negative charge of the ONs and this facilitates condensation of ONs into nanoparticles. Additionally, hydrophobic properties and interactions have also been shown to be involved in the formation and stabilization of the nanoparticles. As compared to covalent strategy, non-covalent approach has been less frequently utilized and the reason for this probably is that most of the CPPs do not enable efficient condensation and/or subsequent delivery of the ON-based cargo and the latter is mainly a consequence of the endosomal entrapment. Nevertheless, lately significant advances have been made in non-covalent vectorization with CPP and many successful reports have been recorded, including the reports that form the basis of this thesis. Pros and cons for covalent conjugation and non-covalent nanoparticle formation with CPPs are described in a Table 2.

Table 2. Overview of the advantages and disadvantages with covalent and non-covalent delivery with CPPs.

Covalent conjugation	Non-covalent nanoparticle formation
Difficult to synthesize/conjugate	Additional synthesizing not necessary
High concentrations required	Low concentrations required
Compatible with uncharged ONs, such as PNA and PMO	Compatible with negatively charged ONs
CPP/cargo conjugates have defined stochiometry	Difficult to generate and characterize homogenous nanoparticles
Not applicable for pDNA and siRNA delivery	Applicable with pDNA and siRNA delivery
Laborious and more expensive	Less laborious and more cost-efficient

1.4.1. pDNA delivery

Peptides have been vigorously used for the vectorization of pDNA. The bottleneck with CPPs for pDNA delivery has been that the relative delivery efficiency has been poor in general [135–137]. The reason for this is mainly originating from the fact that most of the unmodified CPPs do not enable efficient pDNA condensation and even if these particles are taken up they are unable to escape from the endosomes and remain biologically unavailable. Moreover, even if some CPPs show potential *in vitro* few have displayed the same activity *in vivo*, not even in the case of local delivery. To our knowledge there are not many successful reports on the systemic delivery. Additionally, many reports have used CPPs as additives to cationic lipids to enhance their delivery efficiency [135, 138] or they have been used as uptake enhancers for other delivery vehicles [129, 139]. However, as this thesis is concentrating only on CPPs that directly facilitate the nanoparticle formation as single component systems, the combined delivery strategies will not be further described. A selection of examples describing CPP-mediated delivery of pDNA is presented in Table 3.

In early reports, different synthetic peptides were shown to mediate pDNA delivery and enhance transgene expression. For example, Wyman et al. showed that KALA peptide is capable of forming complexes with pDNA and mediating its intracellular delivery [140]. It was hypothesized that the delivery efficiency of KALA peptide was dependent on its ability to form α-helical structures at acidic conditions in the endosomes. In 1997, Morris et al. published a report, that is now considered a proof-of-principle study for non-covalent nanoparticle formation with CPPs, where they showed that MPG peptide formed nanoparticles with shorter DNA fragments and mediated their intracellular delivery [83]. In two years time, they also extended this platform to include the delivery of pDNA [141]. They showed that MPG peptide facilitated the delivery of luciferase-encoding plasmid into various cell lines. Additionally, they showed that

Table 3. Examples of the utilization of CPPs for pDNA delivery

CPP	Gene	Biological effect	References
KALA	Luc	Luciferase expression	[140]
MPG	Luc and cdc25C	Luciferase expression and cell cycle arrest	[141]
Stearyl-Arg8	Luc	Luciferase expression	[104, 130]
ppTG1 and ppTG20	Luc	Luciferase expression *in vitro* and *in vivo*	[142]
Macro-branched Tat	Luc	Luciferase expression	[136]
C-5H-Tat-5H-C	Luc	Luciferase expression *in vitro* and *in vivo*	[127]
POD	RFP	RFP expression	[143]
PEG-POD	Luc	Luciferase and β-galactosidase expression *in vitro* and *in vivo*	[144]
PEG-POD	GDNF	GDNF expression *in vitro* and retinal degeneration rescue *in vivo*	[145]
Stearyl-$(RxR)_4$	Luc and EGFP	Luciferase and EGFP expression	Paper I, [146]
Stearyl-TP10	Luc and EGFP	Luciferase and EGFP expression *in vitro* and luciferase expression *in vivo*	Paper II, [147]
Stearyl-NLS-Arg8	Luc	Luciferase expression	[148]

Luc, Luciferase; RFP, red fluorescent protein; EGFP, enhanced green fluorescent protein; GDNF, glial cell line-derived neurotrophic factor; POD, peptide for ocular delivery; PEG, polyethyleneglycol; stearyl, stearic acid moiety; NLS, nuclear localization signal.

by delivering a pDNA carrying a full-length antisense cDNA encoding human cdc25C, they were able to arrest the cell cycle in large population of cells and prevented their entry to mitosis. In 2002, Rittner et al. designed novel amphipathic CPPs, namely ppTG1 and PPTG20, and evaluated their delivery properties both *in vitro* and systemically *in vivo* [142]. They showed that these peptides enhanced the delivery of luciferase-encoding pDNA in variety of cell lines. Moreover, upon systemic administration of these particles luciferase expression was measured from lung tissues. Unfortunately, these effects were associated with significant toxicity as many animals in the treatment group died because of lung embolism [142]. Lately, Liu et al. designed various macro-branched Tat peptide analogues and showed that they were able to deliver pDNA into variety of cell lines, while transfection efficiency was retained in the presence of serum [136]. Recently, Lo et al. reported a Tat peptide analogue that was modified with histidines and cysteins, generating the C-5H-Tat-5H-C peptide. This peptide complexed with pDNA enhanced luciferase expression in a

variety of cell lines. Moreover, after direct injections to the brain and spinal cord of mice, increased luciferase gene expression was achieved *in vivo* [127].

In 2008, Johnson et al. described a novel peptide-based vector for ocular delivery of pDNA, named peptide for ocular delivery (POD). In this report, they showed that this peptide mediated efficient pDNA transfections *in vitro* [143]. However, this POD peptide was inefficient for *in vivo* delivery and for this it was further modified with polyethylene glycol (PEG), resulting in a PEG-POD peptide [144]. These PEG-POD particles with pDNA were studied for gene delivery *in vivo* after both local and systemic delivery. Strikingly, PEG-POD enabled the increased expression of two transgenes (luciferase and β-galactosidase) after local intraretinal administration. Moreover, after systemic administration of these particles, luciferase expression in lungs was enhanced significantly. Read et al. recently extended the applicability of PEG-POD platform even further and they showed that upon intraretinal delivery of pDNA enconding glial cell line-derived neurotrophic factor (GDNF), enhanced GDNF expression was achieved and it induced functional rescue of mouse retinal degeneration [145].

CPPs modified with fatty acids, especially stearic acid, have been shown to enhance the activity of CPPs for pDNA delivery. For example, Futaki et al. showed that N-terminal stearylation of the octaarginine (Arg8) peptide enable efficient pDNA condensation and gene delivery into wide variety of different cell lines, while the delivery efficiency was reported to be in line with lipofection [104, 130]. However, more recently they have used this stearyl-Arg8 peptide mainly for the functionalization of the MEND to enhance its cellular uptake [129]. Lately, Wang et al. studied the stearylated nuclear localization signal (NLS) modified Arg8 peptide (stearyl-NLS-Arg8) for the pDNA delivery and showed that it increased luciferase gene expression *in vitro* [148]. In conclusion, CPPs have been shown to be able to vectorize pDNA both *in vitro* and *in vivo*, however, despite effective nanoparticle formation capabilities the relative transfection efficiencies have remained poor in general. Therefore, in order to envision the applicability as efficient transfection agents or more importantly for efficient *in vivo* delivery, CPP-based vehicles with significantly improved delivery properties are required.

1.4.2. SCO delivery

Since it was found that asONs can intervene with splicing they have been intensely used to target a variety of diseases associated with incorrect splicing patterns. However, insufficient cellular delivery of these molecules has significanty restricted successful implementation of these molecules for therapeutic purposes. Consequently, CPPs have been very intensely utilized for the delivery of SCOs. Very promising results have been achieved when SCOs, based on PNA and PMO, have been covalently conjugated to the CPP and some of these

conjugates have even been taken into the pre-clinical studies [39]. A selection of examples describing CPP-mediated delivery of SCOs is presented in Table 4.

Early reports showed that covalent PNA conjugates with classical CPPs, such as Tat, penetratin or MAP [149–151], induced significant splicing correction when used at very high conjugate concentrations. Unfortunately, these effects were correlating with high membrane perturbation and toxicity, which allowed the direct penetration across the plasma membrane. Consequently, it became evident that these CPPs are not potent enough to be utilized in such context.

Table 4. Examples of the utilization of CPP for SCO delivery

CPP	ON type	Targeted mRNA	Model	References
Tat, Pen	2′–OMe	Luciferase	HeLa pLuc 705	[149]
Tat	PMO	Luciferase	HeLa pLuc 705	[150]
MAP	PNA	Luciferase	HeLa pLuc 705	[151]
Tat, TP, Pen	PNA	Luciferase	HeLa pLuc 705	[152]
M918	PNA	Luciferase	HeLa pLuc 705	[153]
$(RxR)_4$	PMO	Dystrophin	DMD canine myoblasts, DMD mouse model	[155, 157–159, 163]
Pip peptides	PNA	Luciferase, Dystrophin	HeLa pLuc 705, DMD mouse model	[162]
R6-Pen	PNA	Luciferase	HeLa pLuc 705	[161]
Stearyl-TP10	2′–OMe	Luciferase	HeLa pLuc 705	[131]
Stearyl-$(RxR)_4$	2′–OMe	Luciferase	HeLa pLuc 705	[146], paper I
PF14	2′–OMe	Luciferase, dystrophin	HeLa pLuc 705, DMD myotubes	[164], paper IV
B-MSP	PMO	Dystrophin	DMD mouse model	[165, 166]
P005	PMO	β-globin	β-thalassemia mouse model	[167]

2′–OMe, phospohorothioate 2′–*O*-methyl ONs; PMO, phosphorodiamidate morpholino ONs; PNA, peptide nucleic acid; DMD, Duchenne muscular dystrophy; HeLa pLuc 705, model for splice correction efficiency.

Lately, many groups have intensely studied the possibilities to improve the delivery properties of CPP for the delivery of SCOs. EL Andaloussi et al. showed that transportan and M918 peptide, covalently conjugated to PNA, improved SCO delivery as compared to abovementioned classical CPP/PNA conjugates, however, still high concentrations of conjugates were required to induce significant splicing correction [152, 153]. Another potent peptide-based vehicle was reported, namely $(RxR)_4$ peptide, where polyarginine peptide was modified with 6-aminohexanoic acid linkers (corresponding to x) [154]. Covalent conjugates of $(RxR)_4$ peptide and PMO ($(RxR)_4$-PMO) have been shown to induce significant splicing correction *in vitro*, both in the HeLa pLuc705 model and in disease-relevant DMD canine myoblast model, with the concentration of

conjugates required to obtain 50% exon skipping of the transcript (EC_{50} values) being in the low micromolar range [154–157]. $(RxR)_4$-PMO conjugates have also been successfully used for *in vivo* delivery of SCOs in DMD mouse model, where they have been shown to induce the functional rescue of the dystrophin protein [155, 158, 159]. Moreover, AVI Biopharma has also taken these conjugates into pre-clinical studies, targeting severe viral infections and DMD [155, 160]. Another lately reported peptide is R6-Pen peptide, which is a penetratin analogue that has been elongated with 6 arginine (R) residues in its N-terminus. R6-Pen conjugates with PNA has been shown to reach similar splicing correction efficiency levels that of $(RxR)_4$-PMO conjugates [161]. Recently, another platform was published, namely PNA internalization peptides (Pip peptides), which comprised the elements from both $(RxR)_4$ and R6-Pen peptides and these Pip derivates, conjugated to PNA, were shown to be more efficient in inducing splicing correction than previously mentioned vehicles. Moreover, upon local intramuscular administration *in vivo*, these conjugates also induced efficient rescue of dystrophin in DMD model [162].

In last years there have been significant advances in the field of *in vivo* delivery of CPP/SCO conjugates. While the previously mentioned $(RxR)_4$-PMO induced significant splicing correction in DMD model, enabling the functional rescue of dystrophin in diaphragm and skeletal muscles, these conjugates were unable to induce efficient dystrophin rescue in the heart tissue [159, 163]. Recently a modified $(RxR)_4$ peptide analogue was reported, namely B-peptide, which was further fused with muscle specific heptapeptide (MSP). This peptide conjugated to the PMO showed body-wide dystrophin rescue, leading to the correction of DMD disease phenotype and the restoration of muscle function [165, 166]. Conclusively, all these reports emphasize the utility of covalent conjugates for splice switching therapy.

Lately, our group designed a new potent vehicle for the delivery of SCOs that, to our knowledge, for the first time enabled vectorization of SCOs by non-covalent nanoparticle formation strategy [131]. This peptide, N-terminally stearylated TP10 peptide (stearyl-TP10) was shown to efficiently associate with 2′-OMe SCOs and these particles facilitated significant splice correction *in vitro*. Strikingly, stearyl-TP10/SCO nanoparticles induced splice correction at very low SCO concentrations, compared to all previously mentioned CPP/SCO conjugates. Moreover, when the stearic acid was introduced orthogonally to the 7^{th} lysine in the TP10 sequence, the delivery efficiency was further enhanced [168].

Consequently, there have been substantial successes in inducing splicing correction with covalent CPP/SCO conjugates that have led to many pre-clinical studies. Nevertheless, the potency of these vectors is still not satisfactory and very high doses are required to induce splicing correction. Consequently, there is a considerable interest in creating vectors with improved delivery properties. Recent advances in the non-covalent delivery of SCOs could be one possible solution to enable dose reduction [131].

1.4.3. siRNA delivery

Search for efficient delivery methods for siRNAs has been ongoing ever since it was discovered that synthetic siRNAs were able to induce gene silencing. As a result, CPPs have been vigorously investigated to enhance the delivery of siRNAs, both *in vitro* and *in vivo*. Examples of CPP-mediated delivery of siRNAs are presented in Table 5.

Table 5. Examples of the utilization of CPP for siRNA delivery

CPP	Target mRNA	Effect	References
MPGΔ^{NLS}	Luc/GAPDH	Luciferase and GAPDH downregulation *in vitro*	[134]
MPG8/ Chol-MPG-8	Cyclin B1	Inhibition of the cell proliferation *in vitro*/tumor reduction in mice (Chol-MPG-8)	[169]
CADY	GAPDH	GAPDH downregulation	[99]
Stearyl-Arg8	GFP, MAP2b	GFP and MAP2b downregulation in primary cells	[133]
Stearyl-Arg8 (in MEND)	Luc	Luciferase downregulation	[132]
Stearyl-Arg8 (in MEND)	Luc	Luciferase downregulation in mice	[170]
EB1	Luc	Luciferase downregulation	[126]
Chol-Arg9	VEGF	VEGF downregulation and tumor reduction in mice	[171]
RVG-Arg9	GFP, SOD-1, siFvE^{J}	GFP and SOD-1 downregulation in brain. siFvE^{J} downregulation mediated protection against JEV-induced encephalitis in mice	[172]
Tat-DRBD	dGFP, dRFP, Luc, GAPDH, Oct-4	Efficient gene silencing in "difficult-to-transfect" primary and suspension cells and in mice luciferase model	[173]
Tat-DRBD	EGFR, Akt2	EGFR and Akt2 downregulation induced tumor reduction and increased survival in mice	[174]
PepFect6	Luc, EGFP, HPRT1, Oct-4, GAPDH	Efficient gene silencing in "difficult-to-transfect" primary and suspension cells and in mice upon systemic administration in two *in vivo* models	[175], paper III

NLS, nuclear localization signal; Chol, cholesteryl moiety; MEND, multifunctional envelope-type nanodevice; Luc, luciferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAP2b, microtubule-associated protein 2b; VEGF, vascular endothelial growth factor; (dE)GFP, (destabilized enhanced) green fluorescent protein; SOD-1, superoxide dismutase 1; siFvE^{J}, gene encoding the envelope protein of Japanese encephalitis virus; JEV, Japanese encephalitis virus; EGFR, epidermal growth factor receptor; Akt2, RAC-beta serine/threonine-protein kinase; HPRT1, hypoxanthine phosphoribosyltransferase 1, Oct-4, octamer-binding transcription factor 4.

In 2003, the group of G. Divita reported a first successful attempt to vectorize siRNAs with CPPs [134]. In this, MPGΔ^{NLS} peptide efficiently formed nanoparticles with siRNA and induced significant knockdown of luciferase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This was followed by many papers which utilized both covalent and non-covalent approach to vectorize siRNA. However, after a while it became evident that some of the results in these reports were misleading, as it was shown that if the CPP/siRNA conjugates were delicately purified they failed to confer any biological activity [176, 177]. Therefore, it was believed that, in the successful reports, where conjugates were not purified, probably the excess of CPP formed non-covalent complexes with siRNAs and these mediated the efficient delivery [178, 179]. As a result, it is now believed that a single CPP molecule attached to siRNA is not capable of charge neutralization and does not confer its intracellular delivery, and for this, non-covalent nanoparticle formation approach is most likely the only applicable method to vectorize siRNAs with CPPs. Consequently, the non-covalent nanoparticle approach has been intensely studied for siRNA delivery and many excellent studies have been published.

Kim et al. reported that cholesterol-modified Arg9 was capable of forming nanoparticles with siRNA. By targeting vascular endothelial growth factor (VEGF), these particles were shown to mediate efficient downregulation of VEGF *in vitro*. Moreover, in a tumor xenograft model these particles mediated VEGF knockdown and subsequent tumor reduction *in vivo* [171]. In a very interesting study, Kumar et al. reported targeted delivery of siRNA to the central nervous system using Arg9. In this report, rabies virus glycoprotein (RVG) peptide, a ligand for acethylcholine receptor, was additionally modified with Arg9 to enable siRNA condensation and intracellular delivery. This RVG-Arg9 peptide facilitated efficient nanoparticle formation with siRNA and these particles enabled efficient gene silencing *in vitro*. More importantly, systemic administration of these particles resulted in protection against the fatal viral encephalitis in mice model [172]. In another report, MPG-8 peptide enabled efficient siRNA delivery and, when targeting cell cycle regulator cyclin B1, this resulted in a sequence specific gene silencing and decrease in cell proliferation *in vitro*. Also, these nanoparticles induced significant tumor reduction in a tumor xenograft mouse model upon intratumoral administration. Moreover, if MPG-8 was further modified with cholesterol it mediated efficient tumor reduction and increased the survival of the animals upon systemic administration [169]. Lately, the group of G. Divita reported a new amphipathic CPP-based delivery vehicle of siRNA, namely CADY. This CADY peptide was shown to efficiently form nanoparticles with siRNA targeting GAPDH mRNA and mediated efficient gene silencing in the variety of refractory primary and suspension cells. Unfortunately, no *in vivo* data has yet been reported with this peptide [99].

More recently, the laboratory of S. Dowdy, introduced a very interesting CPP-based platform for the delivery of siRNA using a non-covalent approach

[173]. In this system, CPP motifs of Tat peptide are expressed as a fusion protein with double-stranded RNA-binding domain (Tat-DRBD). This system was shown to enable efficient gene knockdown in various "difficult-to-transfect" cell lines, such as HUVECs and hES cells, without exerting any cytotoxic or immunological side effects. Also, in this publication it was demonstrated that Tat-DRBD/siRNA complexes mediated efficient luciferase gene silencing *in vivo* after local intranasal administrations [173]. Lately, it was reported that Tat-DRBD system allowed tumor reduction in a tumor xenograft model upon intratumoral delivery in mice [174]. Unfortunately, this system is very sensitive to the presence of serum and even to work efficiently i*n vitro* it requires both the removal of glycosaminoglycans (GAGs) from transfection media and utilization of high concentrations of Tat-DRBD. Probably for this reason no systemic delivery with this platform has been reported.

Stearylation of CPPs has been shown to improve the delivery properties of CPPs for siRNAs. Tönges et al. showed that stearyl-Arg8 mediated the delivery of siRNAs to the primary cells, however, the silencing of the expression of EGFP was relatively inefficient [133]. Additionally, stearyl-Arg8 has been used in the MEND platform for surface functionalization to enhance the uptake of these particles. Generally, in the MEND system, core ONs are usually condensed with polylysines or protamines, however, in case of siRNA delivery, these formulations were biologically inactive. Consequently, stearyl-Arg8 was additionally used for the core condensation of siRNAs and these MEND/siRNA particles were shown to mediate luciferase gene silencing both *in vitro* [132] and *in vivo* [170]. Moreover, in these studies they also noticed that stearyl-Arg8 itself, in complex with siRNA and without being formulated with other components of MEND, did not confer biologically relevant gene silencing.

Conclusively, there have been significant advances lately in CPP-mediated delivery of siRNAs. However, there are still many limiting obstacles even *in vitro*. The concentrations of siRNA that is required to induce significant gene silencing with most potent vectors is relatively high and most of these delivery vehicles are very susceptible to the presence of serum proteases. While the number of *in vivo* reports that describe siRNA delivery and gene silencing is increasing, the majority of those have mainly utilized local delivery into tumors and less intriguing information is available for efficient systemic delivery. Therefore, if keeping in mind the potential applicability in clinical settings, there is a significant room for improvements in CPP-mediated siRNA delivery.

2. AIMS OF THE STUDY

The main objective of this work was to design more efficient CPP-based vectors that would form nanoparticles and facilitate the delivery of ONs and their analogues, such as pDNA, SCOs and siRNAs, to induce gene transfer, splicing correction or gene silencing, respectively. Delivery efficiency of these vectors was evaluated in cell cultures, whereas some vectors were also used *in vivo* conditions. In more detail the aims of this study are described below.

- Paper I: To study if modifying $(RxR)_4$ peptide with stearic acid would improve the delivery properties of this peptide and allow this novel stearyl-$(RxR)_4$ to be used for the delivery of pDNA and SCOs by non-covalent nanoparticle formation approach in cell cultures.

- Paper II: To investigate if the previously reported delivery properties of stearyl-TP10 peptide could also be used for the delivery of pDNA in cell cultures, including primary cells. To study if the formed nanoparticles could mediate efficient gene transfer *in vivo* upon local administration regimen.

- Paper III: To evaluate if a novel endosomotropic modification on stearyl-TP10 would yield in a vector with high endosomal escape potential that could be applicable for the cytoplasmic delivery of siRNAs by non-covalent approach. To thoroughly evaluate the potential of this new PepFect6 peptide for the delivery of siRNA in cell cultures, including primary and suspension cells. Moreover, to study if these particles induce gene silencing upon systemic delivery in mice.

- Paper IV: To study if the substitutions in the stearyl-TP10 peptide backbone would improve the stability and delivery efficiency of stearyl-TP10 for SCO delivery *in vitro*. Moreover, to evaluate the delivery potential of this PepFect14 peptide in a disease model of DMD. Finally, to investigate if these nanoparticles could be dried as solid dispersions and retain its efficiency after reconstitution.

3. METHODOLOGICAL CONSIDERATIONS

Materials and methods used in this study are described in detail in each paper, therefore, only general theoretical and methodological aspects will be described in this section.

3.1. Choice of peptides and modifications

In this thesis, several known CPPs, such as $(RxR)_4$, TP10 and Arg9, were used and modified by different means to improve their delivery properties and make them compatible with non-covalent delivery strategy (Table 6).

In paper I, $(RxR)_4$ peptide was chosen since $(RxR)_4$-PMO conjugates have been successfully used for splice correction [154] and these conjugates have reached pre-clinical studies for severe viral infection and DMD [155]. N-terminal stearic acid modification was chosen as the stearylation of polyarginines has been shown previously to enhance the delivery of various ON-based cargos by non-covalent strategy [104, 133].

In paper II, stearyl-TP10 was chosen as it has been previously shown to enable efficient delivery of SCOs by non-covalent strategy [131]. We sought to explore if this peptide could also be used for the delivery of pDNA.

In paper III, the design of the peptide originated form the fact that while stearyl-TP10 was very efficient for the delivery of cargos that eventually had to reach to the nucleus, such as pDNA and SCOs [131, 147], it was not compatible with cytoplasmic delivery of siRNAs (unpublished observations). Therefore, to further induce rapid endosomal escape, we incorporated a novel endosomotropic modification – trifluoromethylquinoline – that would mimic the proton-accepting properties of chloroquine. Four of these modifications, on a succinylated lysine tree, were attached to the 7^{th} lysine of TP10 peptide, producing an efficient vector for siRNA delivery (PepFect5). Unfortunately, its efficiency was severely affected by the presence of serum. Consequently, based on our previous knowledge of the increased serum stability upon stearylation [131], this peptide was further modified with stearic acid, generating a PepFect6 peptide.

In paper IV, in order to enhance nanoparticle formation efficiency and serum stability of stearyl-TP10, we modified the peptide sequnce of stearyl-TP10. For this, ornithines were introduced instead of lysines as a source of positive charges. Ornithines were chosen because it had been previously reported that poly-L-ornithines have superior delivery properties as compared to poly-L-lysines [180]. Moreover, we hypothesized that ornithines as a non-standard amino acid should be less susceptible to the serum proteases.

Finally, several modified and unmodified CPPs were used as controls in all of the papers. Moreover, different commercially available cationic lipids and polycations were used for comparison.

Table 6. CPP sequences used in the thesis.

CPP	Sequence	Ref.
$(RxR)_4$	RXRRXRRXRRXR-NH_2	[181]
stearyl-$(RxR)_4$	stearyl- RXRRXRRXRRXR-NH_2	[146]
Arg9	RRRRRRRRR-NH_2	[182]
stearyl-Arg9	stearyl-RRRRRRRRR-NH_2	[131]
TP10	AGYLLGKINLKALAALAKKIL-NH_2	[94]
stearyl-TP10, PepFect3, PF3	stearyl-AGYLLGKINLKALAALAKKIL-NH_2	[131]
PepFect5, PF5	AGYLLGK[a]INLKALAALAKKIL-NH_2	[175]
PepFect6, PF6	stearyl-AGYLLGK[a]INLKALAALAKKIL-NH_2	[175]
PepFect14 A, PF14 A	AGYLLGKLLOOLAAAALOOLL-NH_2	[164]
PepFect14, PF14	stearyl-AGYLLGKLLOOLAAAALOOLL-NH_2	[164]

Stearyl, stearic acid moiety; X, 6-aminohexanoic acid.
[a] four trifluoromethylquinoline moieties attached to a succinylated lysine tree

3.2. Peptide synthesis

Solid phase peptide synthesis (SPPS) is a method for peptide synthesis that was introduced by Bruce Merrifield in 1963 [183]. The general principle of SPPS is that synthesis is carried out on an insoluble polymer, a resin, where N^{α} protected pre-activated amino acids are coupled on the resin in a stepwise manner. After a series of deprotections and couplings all the protecting groups are removed, synthesized peptide is cleaved from the resin and free peptide is obtained. Generally SPPS is based on two main chemical strategies – *tert*-butyloxycarbonyl (*t*-boc) and 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. In this thesis both of these chemistries have been utilized.

All the peptides used in this thesis were synthesized by either Fmoc or *t*-Boc chemisty using rink-amide methylbenzylhydrylamine-polysterene (MBHA) resin to generate C-terminally amidated peptides. After the final cleavage, peptides were purified using reversed-phase high performance liquid chromatography (RP-HPLC) and the mass of the peptides was measured using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

3.3. Non-covalent nanoparticle formation between CPPs and ONs

As aforementioned, non-covalent nanoparticle formation between CPPs and cargo was first demonstrated by the group of F. Heitz and G. Divita [83]. While first proposed for DNA and pDNA [83, 141] this approach can also be used for the delivery of many other molecules, including peptides, proteins, siRNA and SCOs [95, 131, 134]. CPPs form nanoparticles with ONs predominantly by electrostatic and hydrophobic interactions. Consequently, most of the CPPs that

have been successfully used for ON delivery are usually positively charged and are partially amphipathic [184]. A general rule for the condensation of negatively charge ONs is that at least charge neutralization is required for the nanoparticle formation.

Nanoparticles between CPPs and different ON-based cargo, i.e. pDNA, SCOs and siRNAs, were formed in the similar manner. Briefly, ONs were diluted with MQ water followed by the addition of CPP solution. Thereafter, components were simply mixed and the mixture was incubated for 30 to 60 min at the room temperature for the spontaneous nanoparticle formation. The CPP amount over ONs in all the studies was carefully titrated in order to find the most optimal conditions. This process is crucial, as optimal condition can significantly vary between the different delivery vehicles and delivery conditions *in vitro* (presence of serum for instance), whereas the picture is even more diverse in *in vivo* conditions. For general overview of the CPP/ON nanoparticle formation, see Figure 4.

Additional considerations must be taken into account in case of different ONs used in this thesis, e.g. pDNA, SCOs and siRNAs. First, pDNA is a very large molecule that carries thousands of negative charges. For example, the 4.7 kbp pGL3 plasmid has 9400 negative charges and the amount of peptide needed to neutralize these charges to enable nanoparticle formation is significantly higher than in case of shorter ONs. Consequently, even at the theoretical equilibrium ratio of charges (that is referred to as charge ratio 1, CR1, in the papers) the amount of peptide molecules have to be in thousands. However, for shorter ONs these parameters are different. For instance, single-stranded SCOs usually require 3–10 peptide molecules per ON (referred to as molar ratios, MRs, in the papers). In case of double-stranded siRNAs the amount of negative charges is higher and, therefore, usually around 30–40 peptide molecules per siRNA molecule is needed (referred to as molar ratios, MRs, in the paper). Also, see Figure 4.

In case of *in vivo* formulations, additional aspects have to be taken into account. It is generally known that correlating *in vitro* results with *in vivo* findings is often impossible. Our experience is that higher ratios of peptides over ONs are often very efficient *in vitro*, however, these particles often tend to be inefficient *in vivo*. We hypothesize that avidity of the vehicle towards the cargo is too great to enable efficient dissociation of cargo upon intracellular delivery *in vivo*. Therefore, we have utilized lower CPP amount over ONs with *in vivo* delivery, namely paper II (CR1) and III (MR30). Additionally, the amount of both CPP and ON used for *in vivo* formulations need to be at least an order of magnitude higher than for cellular treatments. As the volume that can be injected into mice usually cannot exceed certain volumes (approximately 25 µl for intramuscular and intradermal and 200 µl for intravenous injections), these particles have to be formulated at a substantially higher stock concentration. However, at these high concentrations aggregation and sedimentation of the particles can be experienced, which also limits the dose-ranges that can be

used. Therefore, for *in vivo* formulations we used additional additives, isotonic solutions of glucose [185] or mannitol [186], which have been shown to enhance the colloidal stability of nanoparticles, crucial for the *in vivo* applicability and reproducibility. Moreover, parenteral injection vehicles in general have to be isotonic not to cause stress to the subjects of the study.

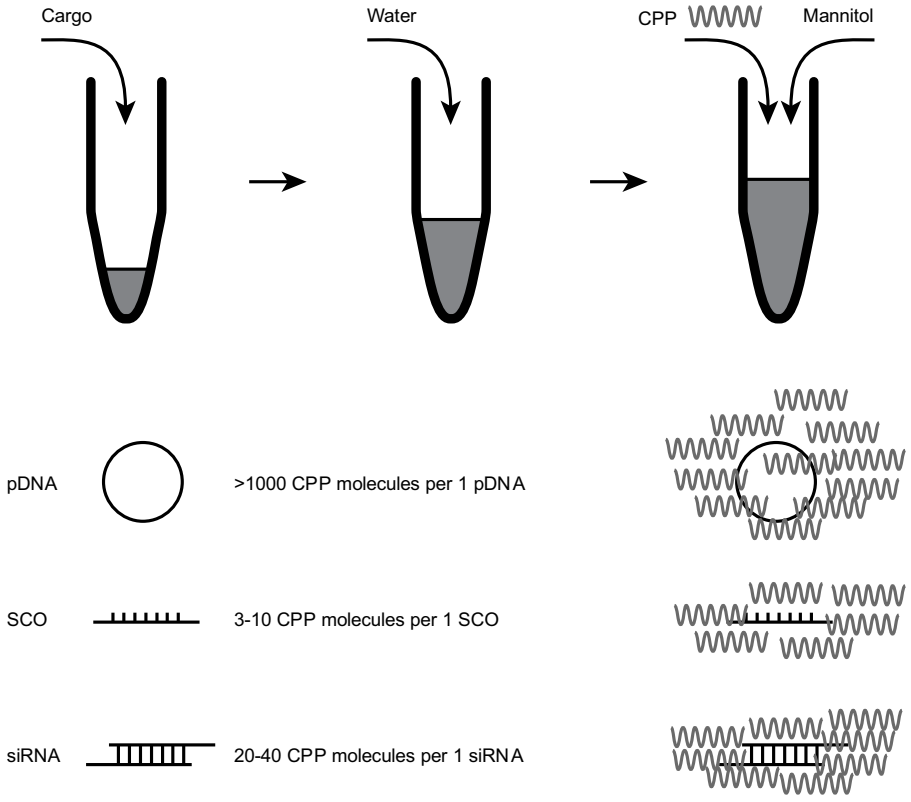

Figure 4. Overview of the protocol for the formation of CPP/ON nanoparticles. ONs are diluted with water and then incubated with CPPs for the spontaneous nanoparticle formation. Depending on the charge distribution of cargo, different amount of cationic CPP is required in order to neutralize anionic ONs and enable the particle formation. In case of *in vivo* formulations, additives, such as mannitole or glucose, are added after the formation of the particles to stabilize the system and make it isotonic.

3.4. Nanoparticle characterization

It is well known that the delivery efficiency, biodistribution and side-effects of nanoparticles are closely related to their physicochemical properties. There is a variety of methods that could be utilized to gain insight into these properties the approaches used in this thesis will be described below.

3.4.1. Gel shift assay and serum stability

Gel shift assay is the easiest and most well-known way to determine if the vector is capable of forming complexes with the cargo. The formed particles are incapable of migrating in the gel during electrophoresis due to their size. This assay can be easily extended to assess the serum stability of the particles. We have utilized this assay in the paper I and II.

3.4.2. Ethidium bromide quenching

Ethidium bromide (EtBr) exclusion is a simple assay to evaluate the complex formation efficiency. The principle of this method is based on the EtBr ability to increase its fluorescence upon binding to nucleic acids. Therefore, if the nucleic acids are efficiently shielded by the condensing agent, almost no EtBr can bind to the complexes resulting in the quenching of fluorescence. We have used this assay in papers I and II.

3.4.3. Dynamic light scattering (DLS) and zeta potential measurements

Particle size and homogenicity are crucial parameters that determine the delivery efficiency. Even though particles with different size often work for *in vitro* transfections, the situation is often much more complicated for the *in vivo* conditions. For example, particles with a certain size may target different organs and have different tissue distribution profiles. In this context, obtaining particles with a defined size might be crucial.

DLS is a method that enables the determination of particle size and distribution. This technique has been used in all four publications for the evaluation of particle size. These parameters have also been measured after the addition of serum supplements to provide a better understanding of what might happen under physiological conditions.

Another important parameter that can be subsequently measured by the same methodology is the particle surface charge (zeta potential). In general, for *in vivo* applicability particle surface charge should remain in the range of +30 and –30 mV. Surface charge determines how fast the particles undergo opsonisation and are cleared from the circulation. We have conducted these measurements, in the papers II, III and IV.

3.4.4. Heparin displacement assay

For particle formation the delivery vehicle must condense the cargo molecules. However, for the activity of the cargo at its active site, it is equally important that these vector/cargo complexes are dissociating. Heparin displacement assay

is a simple methodology for the estimation of avidity of a vector towards the cargo. In principle, heparin sodium salt is an anionic molecule that has a greater binding affinity than ONs towards the vector. Consequently, the concentration of heparin needed to dissociate the vector/cargo complex gives an indication of the particle stability. This phenomenon can be registered by gel electrophoresis. In Paper II we have utilized this assay for the determination of the stability stearyl-TP10/pDNA nanoparticles.

3.4.5. Liposome leakage assay

Endosomal entrapment has been established as the most crucial factor that limits the bioavailability of CPPs. The ability of a vector to escape from the endosomes is widely associated with its intrinsic capability of disrupting the endosomal membranes. Many strategies that aim to increase endosomal escape are based on the so-called "proton sponge" effect. In this case vehicles are designed in a pH-sensitive manner. Upon ionization at the lower pH of endosomes these molecules attract protons (and counter ions) into the vesicles. Consequently, osmotic pressure is increased that cause the swelling and rupture of the vesicle. Another advantage of this method is that buffering of the endosomal pH probably delays the lysosomal degradation.

The liposomal leakage assay used here is an artificial system based on liposomes that mimics the membrane composition and pH of late endosomes. Upon membrane perturbation a quenched fluorophore incorporated inside the liposomes leaks into surrounding media and can be measured by the means of fluorescence. Therefore, delivery vehicles with endosomotropic properties should induce the efflux of the fluorophore, consequently providing insight into the endosomotropic properties. We have used this assay in paper III.

3.4.6. Solid dispersion technique and stability studies

Solid dispersion is a technique that is widely used in the pharmaceutical industry for the formation of powder to tablets, capsules, sustained-release, inhalation and injection formulations. We have taken advantage of this approach to understand whether CPP-based nanoparticles can be formulated in this way and thus have additional information if these particles could be used as pre-formed formulations or in pharmaceutical dosage forms. In paper IV, we formed solid dispersions of CPP/SCO nanoparticles by the addition of lactose monohydrate, mannitol or PEG6000 to the formed complexes and dried the mixture at elevated temperatures in a DNA speed-vac. To evaluate the applicability of such approach, we reconstituted the solid dispersion in MQ water and carried out the transfections as with regular nanoparticles.

Stability of the nanoparticles is crucial for understanding at which conditions they could be stored and whether they are stable enough to be applicable *in*

vivo. In paper IV we studied the stability of the CPP/cargo nanoparticles that were formed according to the standard protocol or as solid dispersions over the course of 8 weeks. Moreover, we carried out the accelerated stability test by keeping the formed particles at increased temperature over a long time periods.

3.5. Cell cultures

In this thesis more than 25 different cell lines have been used and the choice of the cell lines is described very briefly here. For the overview of the cell lines used in this thesis, see Table 7.

In papers I and II, the delivery efficiency of CPP/pDNA nanoparticles was evaluated in different adherent cell lines that are commonly used for the screening of potential delivery vehicles, while in paper II, we additionally used more refractory primary mouse embryonal fibroblasts (MEFs). See Table 7.

In papers I and IV, the delivery efficiency of CPP/SCO nanoparticles was evaluated in the most widely utilized assay for splice correction, making use of the so-called HeLa pLuc705 cell line (Figure 5) [187]. These cells are stably transfected with luciferase-encoding gene interrupted by a mutated intron 2 from β-globin gene carrying a cryptic splicing site. Incomplete removal of the intron from the pre-mRNA gives rise to the aberrant and non-functional luciferase. Masking the cryptic splice site with SCO enables the redirection of the splicing and the production of functional luciferase. It is a very advantageous model as it provides a positive read-out over a low background while having a wide dynamic range.

Importantly, in paper IV, we evaluated splicing correction efficiency in a disease-relevant *in vitro* model of DMD. In this leading DMD model, H2K mdx mice myotubes carry a point mutation in exon 23 of dystrophin gene. Masking of the mutation site in the pre-mRNA with SCOs mediates the redirection of splicing by inducing exon skipping, restoration of the reading frame and the production of a semi-functional dystrophin (see also Figure 5).

In paper III, we evaluated the siRNA mediated gene silencing in a large battery of cells. For initial screening, we used a variety of reporter cell lines, stably expressing either luciferase or EGFP. More importantly, to estimate the true potential of PF6/siRNA nanoparticles, we also used a wide-range of different refractory cells, including primary and suspension cells. List of the used cells and cell lines can be found in Table 7.

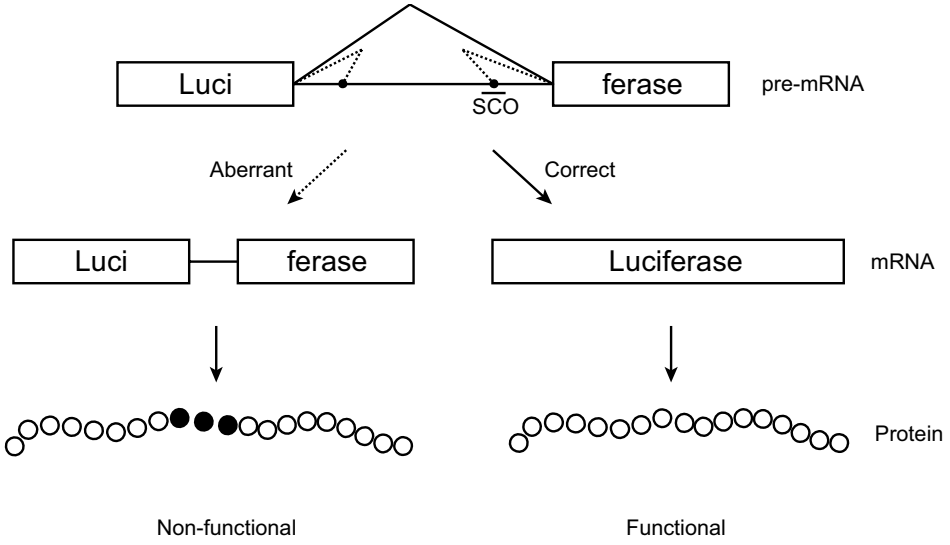

Figure 5. HeLa pLuc705 reporter system for the evaluation of splicing correction efficiency. Luciferase gene is interrupted by the mutated β-globin intron at the nucleotide position 705 that produces incorrectly processed pre-mRNA. Masking this site by SCOs redirects the splicing towards the mRNA and corresponding protein that is functional.

Table 7. Different cells and cell lines used in this thesis.

Cells and cell lines		
Paper I	pDNA	Regular adherent cells: CHO, BHK21, HEK293
	SCO	Reporter cells: HeLa pLuc705
Paper II	pDNA	Regular adherent cells: CHO, U2OS, U87, HEK293 Refractory cells: MEF
Paper III	siRNA	Reporter cell lines: Luc-HEK, Luc-HeLa, Luc-BHK21, Luc-U2OS, Luc-HepG2, EGFP-CHO Refractory cells: N2a, SHSY5Y, B16, Hepa1c1c7, RD4, glia, Jurkat, K562, MEF, HUVEC, C17.2, mES
Paper IV	SCO	Reporter cells: HeLa pLuc705, H2K

HEK, human embryonic kidney; HeLa, human cervical cancer; BHK21, hamster kidney; U2OS, human osteosarcoma; CHO, hamster ovarian; U87, human glioma; MEF, primary mouse embryonal fibroblasts; Luc, luciferase; EGFP, enhanced green fluorescent protein; N2a, mouse neuroblastoma; SHSY5Y, human neuroblastoma; B16, mouse melanoma; Hepa1c1c7, mouse hepatoma; RD4, human skeletal muscle; glia, rat primary glia; Jurkat, T lymphocytes; K562, human myeloid leukaemia; HUVEC, human umbilical vein endothelial cells; C17.2, mouse neuronal stem cells; mES, mouse embryonal stem cells; H2K, H2K mdx mice myotubes.

3.6. Evaluation of the delivery efficiency of CPP/ON nanoparticles

CPP-mediated delivery efficiency of pDNA, SCOs and siRNAs was evaluated by different means, which will be described in the context of particular effector molecules. Generally, for the evaluation of delivery efficiency, we have mainly used techniques that are based on different reporter systems, such as luciferase and EGFP. Also, we have used well established methods based on reverse-transcription polymerase chain reaction (RT-PCR) to measure the changes in gene expression on the mRNA levels.

3.6.1. pDNA delivery

In papers I and II we have mostly used different pDNAs which express firefly luciferase. Luciferase is an enzyme that catalyzes the conversion of luciferin into oxyluciferin and consequently produces bioluminescence, which can be measured by luminometer. This method is the easiest and most utilized end-point measurement for estimating the delivery efficiency of the pDNA. Moreover, it is also a positive read-out assay since plasmid needs to reach the nucleus and be transcribed there.

Additional information can be obtained by using EGFP-encoding plasmid. EGFP is a protein with fluorescent properties and this could be detected by different techniques, including fluorimetry, fluorescence or confocal microscopy and/or flow cytometry. It allows, similarly to luciferase, simple end-point measurements. However, the main difference is that by measuring fluorescence of individual cells or by visualizing the transfected cells by confocal microscopy it is possible to evaluate how many cells from the transfected cell population exactly produce EGFP and whether the expression levels differ from cell-to-cell. Flow cytometry enables the evaluation of these effects even more precisely. We have used confocal microscopy in paper I and II.

To evaluate the vectors for gene delivery *in vivo* we utilized the luciferase reporter system since it allows live animal bioluminescent imaging in mice. It works according to the same principle as described above for *in vitro* measurements of luciferase expression, except that animals are injected with luciferin. In paper II, we have evaluated the stearyl-TP10-mediated pDNA delivery efficiency *in vivo* upon local administration of these particles into the muscle or skin and analyzed it by bioluminescence imager.

3.6.2. SCO delivery

To evaluate the splice correction efficiency we have throughout the paper I and IV used the abovementioned assay based on HeLa pLuc705 cell line (see Figure 5). In this system, upon nuclear delivery, SCOs can mask the aberrant splicing

site and redirects the splicing of pre-mRNA with concomitant production of functional luciferase. This has been used throughout the paper I and IV.

The luciferase-based method does not directly allow the estimation of how much of the transcript has been corrected but merely provides information about the relative efficiency between different vectors. To quantitatively assess the levels on splice correction, we have used RT-PCR. For RT-PCR, cellular pool of mRNA is extracted and used as a matrix for the synthesis of complementary DNA (cDNA). As a next step, by using specific primers, cDNA is used as a template for regular PCR. In case of HeLa pLuc705, the corrected mRNA is shorter than the aberrantly spliced one, and by using RT-PCR, the conversion rate of the mRNA can be measured, indicating the true splicing correction efficiency. We have used RT-PCR also in the DMD model of H2K mdx mice myotubes, where the exon 23 skipping shows the splice correction efficiency. This method was used in the paper IV.

3.6.3. siRNA delivery

The easiest way to initially assess siRNA-mediated RNAi when evaluating new delivery vectors is to use reporter cell lines that have been stably transfected with reporter genes enabling easy read-outs, for example luciferase or EGFP. Methodological considerations for measuring RNAi-mediated gene silencing in paper III will be described below.

Downregulation of luciferase expression with siRNAs in luciferase-encoding cell lines is a good reporter system for end-point measurements. By measuring the decreased luciferase activity by luminometer and normalizing it against the luciferase expression values obtained from control cells, gene silencing can be easily estimated.

Analogously to luciferase, downregulation of EGFP with siRNAs in EGFP-stable cell lines can also be used for the end-point measurements by measuring the fluorescence values. More importantly, targeting EGFP can also give additional information. Measuring the expressed EGFP by flow cytometry and confocal microscopy provide qualitative data to what extent the EGFP is expressed in a cell populations and whether the expression levels differ from cell-to-cell. Flow cytometry is a method that gives extra information concerning the transfection efficiency, as it enables estimation of the cell populations that are stably expressing EGFP and therefore the shift in the decrease of gene expression can more specifically assigned.

Reporter genes are typically integrated in very active regions of the genome with high expression. Therefore, targeting more relevant endogenous or disease-associated genes is a logical step forward. We chose to target hypoxanthine phosphoribosyltransferase 1 (HPRT1), a gene that is expressed in all cells and is well conserved between species, thus it allows the utilization of one universal siRNA. Moreover, the HPRT1 protein has a relatively long half-life (~48 h), which means that the knockdown of mRNA should have a minimal impact on

the viability of the cells within 24 h, the time point we measured RNAi responses. Additionally, HPRT1 siRNA was designed to have minimal off-target effects [188]. HPRT1 mRNA levels were analyzed either by RT-PCR or quantitative RT-PCR (RT-qPCR) and normalized against the levels of internal standard GAPDH. Moreover, HPRT1 knockdown was also confirmed in HeLa cells by using microarray and proteomics techniques that are described below. For mES cells we also designed a siRNA against the pluripotency regulator, Oct-4 [173]. RNAi response of Oct4 mRNA was measured by RT-qPCR and the functional consequence of knockdown was assayed by monitoring loss of pluripotency of the mES-cells.

To evaluate RNAi in mice, we used two different *in vivo* models. First we targeted the endogenous HPRT1 gene analogously to the *in vitro* evaluations. Animals were injected with PF6/HPRT1-siRNA nanoparticles, tissues were dissected and HPRT1 mRNA levels in samples were analyzed by RT-qPCR using GAPDH as an internal standard. In the second *in vivo* model mice livers were stably transfected with a luciferase-encoding plasmid by hydrodynamic technique, which enables stable expression of luciferase over long periods of time. When the transgene expression had stabilized, we treated animals with PF6/luc-siRNA nanoparticles and measured the knockdown of luciferase by *in vivo* bioluminescence imaging.

3.7. Evaluation of toxicity

Toxic and immunological side effects are the main reasons why most of the drugs and delivery platforms never become exploited in clinical settings. To exclude the toxic and/or immunological side effects that could be associated with the particular treatments it is crucial to carry out at least primary evaluation of these parameters *in vitro*. Moreover, in case of *in vivo* delivery, thorough evaluation of the potential toxic and immunological side effects is a pre-requisite.

3.7.1. Cytotoxicity

The easiest way to evaluate the cytotoxicity of different compounds is to use cell viability assays that measure the metabolic activity of the mitochondria. To determine the impact of the transfections on the viability of the cells we have utilized either MTS or WST-1 assay in all four papers.

3.7.2. Microarray and proteomics analysis

Full genome microarray analysis is a methodology that could be used to estimate the impact of the transfection reagents on the transcriptome. We used

this technique to evaluate the off-target effects imparted by the transfection with PF6/siRNA and lipofection, and to have a further indication that the specific genes have been silenced on the mRNA level. Additionally, it is known that mRNA levels do not necessarily correlate with protein levels. Therefore, we assessed the effect of the transfections on the proteome by mass-spectometry-based proteomics. We have used these techniques in paper III.

3.7.3. Toxicity and immunogenicity

To have an indication whether a therapeutic entity has a potential to be used in a clinical setting it is of outmost importance to have an understanding if the *in vivo* administrations are associated with any toxic side effects. To assign this, we have, in paper II and III, conducted thorough evaluation of various parameters. We measured common clinical chemistry and haematology parameters including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels – indicators of liver toxicity (in papers II and III); creatinine level – reflecting renal toxicity (in papers II and III); C-reactive protein (CRP) – indicator of systemic inflammation (in paper III), red and white blood cell count – a common haematological parameters (in paper III). To corroborate the clinical chemistry parameters, we also carried out histopathological examination of the investigated organs and tissues (paper II and III). Additionally, we measured acute toxicity, such as the change of body-weight loss and spleen weight (in paper III).

Another important parameter for *in vivo* applicability is that the *in vivo* treatment would not be associated with the triggering of immunological responses. To evaluate this, we measured the levels of the key cytokines, namely IL-1β (*in vitro*), TNF-α (*in vitro* and *in vivo*) and IL-6 (*in vivo*), by enzyme-linked immunosorbent assay (ELISA). We used these assays in papers II and III.

4. RESULTS AND DISCUSSION

The papers included in this thesis describe the development and characterization of different CPP-based vectors that non-covalently form nanoparticles with ON-based molecules, i.e. pDNA, SCOs and siRNAs, and facilitate their delivery *in vitro* and *in vivo*. In the paper I, we stearylated the pre-clinically used $(RxR)_4$ peptide to enable nanoparticle formation with pDNA and SCOs and studied the delivery efficiency of these particles *in vitro*. In papers II, III and IV, TP10 peptide was subject to different modifications. In paper II, we used the previously reported stearyl-TP10 vector for efficient gene transfer *in vitro* and locally *in vivo*. In paper III, we introduced a novel endosomotropic modification to the stearyl-TP10 peptide and studied this new vector, PepFect6, for siRNA delivery *in vitro* and after systemic delivery *in vivo*. In paper IV, we assessed how replacement of amino acids in the stearyl-TP10 backbone resulted in a more stable and efficient delivery vector for SCOs *in vitro*.

4.1. Stearyl-$(RxR)_4$ peptide-mediated delivery of pDNA and SCOs

CPPs have been successfully used for the delivery of both pDNA and SCOs. Among these reports, $(RxR)_4$ peptide has been shown to be one of the most efficient vectors for the delivery of SCO (SSOs) upon covalent conjugation to PMOs [154, 156] and these conjugates have also reached pre-clinical trials [155]. It has been previously reported that the addition of stearic acid modification to the CPPs, including arginine-rich peptides, enhances their delivery properties and makes them compatible with non-covalent delivery [104, 133]. Our aim was to modify this $(RxR)_4$ peptide with stearic acid and to investigate whether this new $(RxR)_4$ peptide analogue would be applicable for SCO and pDNA delivery by a non-covalent approach.

Stearyl-$(RxR)_4$ efficiently formed nanoparticles with pDNA, with a size of around 370–450 nm, and these particles facilitated efficient gene delivery of luciferase-encoding pDNA and increased the luciferase expression significantly in a variety of cell lines, including CHO, BHK21 and HEK293 cells. Delivery efficiency of stearyl-$(RxR)_4$ was also compared with stearylated polyarginine, as they have been shown to mediate pDNA delivery [104], and while it also enabled pDNA delivery to some extent, it was significantly less efficient than stearyl-$(RxR)_4$. To really appreciate the delivery efficiency of this vector, we made comparisons with the most widely used commercially available transfection agent based on cationic lipids – Lipofectamine 2000 (LF2000). As compared to LF2000, delivery efficiency of stearyl-$(RxR)_4$ was more than a log lower. However, it is well known that transfection efficiency of LF2000 widely correlates with its toxicity [189]. Evaluation of cytotoxicity showed that while lipofection reduced the cell viability by 30%, $(RxR)_4$/pDNA particles did

display no toxicity. LF2000 is also known to transfect cells non-uniformly, i.e. LF2000 enables extremely high transfection levels in some populations of the cells, whereas some remain completely non-transfected. In opposite, although overall transfection efficiency of stearyl-$(RxR)_4$ was lower than with LF2000, it mediated uniform transfections in whole cell populations.

After confirming the delivery potential of stearyl-$(RxR)_4$ for pDNA, we sought to investigate its delivery potential for SCOs to induce splice correction. To evaluate this we used the functional splice correction assay in HeLa pLuc705 cells [187]. In this reporter system, stearyl-$(RxR)_4$/SCO particles mediated efficient SCO delivery and splice correction up to 20-fold. We compared these effects with aforementioned $(RxR)_4$-PMO covalent conjugates [154] and carried out dose-response treatments for both regimens. Strikingly, stearyl-$(RxR)_4$/SCO nanoparticles allowed comparable splicing correction levels at 10-times lower SCO concentration. We also made additional comparisons with LF2000 and stearyl-TP10, previously reported vector from our group that was shown to vectorize SCO non-covalently [131]. Stearyl-$(RxR)_4$ did not quite reach the delivery efficiency of these vectors, which were roughly twice as efficient.

It is complicated to answer why exactly stearyl-$(RxR)_4$ is compatible with non-covalent delivery. It can be hypothesized that addition of stearic acid renders the peptide more amphipathic, which should assist with both nanoparticle formation and delivery efficiency. For example, CPP-based delivery vehicles that have been used for non-covalent delivery are almost exclusively amphipathic [184]. Another clear finding is that these particles are taken up by endocytic pathways and probably stearylation also increases the membrane activity on endosomal membranes, which allows some of the particles to escape from the vesicles. The latter has been indicated recently by finding from our collaborators who have seen that stearyl-$(RxR)_4$ induces membrane perturbation in the liposome leakage assay (that mimics endosomes) and this supports the partial endosomal escape theory (Hassane et al., unpublished observations).

Conclusively, stearyl-$(RxR)_4$ was capable of mediating the delivery of pDNA and SCOs and induced efficient gene transfer and correction of splicing, respectively. In case of SCO delivery, it is, to our understanding second vector, besides the stearyl-TP10, that has been shown to be compatible with non-covalent delivery. Moreover, it is more potent than the most efficient vectors available at the moment, for example $(RxR)_4$-PMO conjugates. Finally, it would be interesting to see if these promising *in vitro* results could be extrapolated to *in vivo* conditions.

4.2. Stearyl-TP10-mediated delivery of pDNA in vitro and in vivo

Our group recently reported a stearyl-TP10 vector for the delivery of SCOs using a non-covalent nanoparticle formation approach [131]. In paper II, we investigated the possibilities of extending this platform for the delivery of pDNA *in vitro* and for local *in vivo* delivery.

Stearyl-TP10 efficiently formed nanoparticles with pDNA as confirmed by different techniques and these particles had an average size of 110–130 nm with a surface charge of around –5 to –11.4 mV. These particles mediated efficient intracellular delivery of luciferase-encoding pDNA in a variety of adherent cell lines, including CHO, U2OS, U87 and HEK293, producing around 3–4 order of magnitude increases in luciferase expression. Also, we carried out transfection in the presence of serum and stearyl-TP10 retained most of its activity under those conditions, which is very important for potential *in vivo* applications. Strikingly, around 2.5 log increase in luciferase expression was also recorded in primary MEF cells, which are significantly more refractory as compared to regular cell lines. We also made comparison with LF2000 and the efficiency of stearyl-TP10 was almost in line with LF2000. However, transfections with stearyl-TP10 were not associated with any adverse effects on cell viability as opposed to lipofection. Stearyl-TP10 fulfilled many other criteria of being an efficient transfection agent *in vitro*, namely: transfection of whole cell population in a uniform manner; non-toxic nature; insensitivity to serum proteases; delivery of pDNA with different size; and relative independence of cell confluence.

Next we investigated the gene delivery potential of stearyl-TP10 *in vivo*. We administered stearyl-TP10/pDNA particles into muscle or skin by intramuscular and intradermal injections, respectively. These treatments induced substantial luciferase expression in these tissues in dose-dependent manner. It is well known that naked plasmid *per se* can mediate significant transgene expression when directly injected, for example into muscles [190]. However, stearyl-TP10/plasmid nanoparticles enhanced this effect by another order of magnitude. This is a very encouraging result, as these effects are in line with results obtained with the most efficient non-viral delivery platforms for intramuscular delivery [191–193]. Finally, we also confirmed that treatments with stearyl-TP10/pDNA nanoparticles were not associated with the induction of immune responses *in vivo* and that treatments were not associated with local or systemic toxicity.

Conclusively, stearyl-TP10 is a very interesting delivery vector for pDNA, both as transfection agent *in vitro*, but more importantly, as a vector for pDNA delivery *in vivo*.

4.3. PepFect6-mediated delivery of siRNAs in vitro and in vivo

CPP-mediated delivery of siRNAs and induction of gene silencing by RNAi has been reported numerous times. We have tried a battery of CPP-based vectors for siRNA delivery, also the ones previously described in this thesis, however, they have all failed to confer reasonable RNAi responses when being non-covalently complexed with siRNAs (unpublished observations, [194]). Our results indicate that the reason for this is that these complexes are sequestered in the endosomes. To overcome this limitation we designed a novel endosomotropic modification – a trifluoromethylquinoline moiety – and we conjugated four of these molecules to the TP10 peptide. This peptide (i.e PepFect5) was very efficient for siRNA delivery *in vitro*, however, only in media devoid of serum proteins. Bearing our previous results in mind that a stearyl moiety increases serum stability of TP10, we further modified this peptide N-terminally with a stearic acid moiety and this resulted in the novel PepFect6 (PF6) peptide.

PF6 efficiently condensed siRNAs into nanoparticles with an average size of around 70–100 nm with a slight negative surface charge (–7 to –11 mV). These PF6/siRNA nanoparticles mediated efficient siRNA-mediated gene silencing in a variety of reporter cell lines targeting luciferase/EGFP mRNA with IC50 values ranging from 2.8 to 8.9 nM in HEK, HeLa, U2OS, CHO, RD4, Hepa1c1c7 and BHK21 cell lines.

After the initial screening in reporter cell lines, we sought to evaluate the potential of PF6/siRNA for silencing of an endogenous target, namely HPRT1, and investigate the gene silencing potential of PF6 in more refractory primary and suspension cells. PF6/siRNA nanoparticles induced significant gene silencing in primary cells, such as MEFs and HUVECs. Also similar effects were achieved in a very "difficult-to-transfect" Jurkat suspension cell line. In these cells, cationic lipids, LF2000 and Lipofectamine RNAiMAX (RNAiMAX), did not allow any significant gene silencing. As result we included a new reagent for comparison, Tat-DRBD (Transductin). This reagent has recently been reported as a new efficient measure for the delivery of siRNAs [173], and fronted the June 2009 issue of Nature Biotechnology. While Tat-DRBD also mediated siRNA-induced gene silencing, it was significantly less efficient than PF6. Finally, we targeted mES cells, where PF6/siRNA nanoparticles induced substantial gene silencing, exceeding the efficiency of all the other transfection reagents. We also confirmed that PF6 did not affect the pluripotency state of mES cells. Moreover, when using siRNA targeting pluripotency regulator Oct-4, we were able to register significant downregulation of Oct-4 mRNA and this knockdown resulted in the loss of pluripotency of mES cells.

Strikingly, PF6 seems to fulfill all the criteria for a very efficient transfection agent: it is not significantly hampered by the presence of serum; its transfection efficiency is relatively independent of cell confluence; it transfects entire cell populations; mediates rapid and persistant RNAi responses over several days;

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and enables complete mRNA and protein knockdown in multiple treatment trials. Taken together, PF6 is substantially more effective than most efficient commercial siRNA transfection agents, i.e. cationic lipids, such as LF2000 and RNAiMAX, and the recently reported Tat-DRBD.

We also assessed the *in vitro* toxicity and immunogenicity profile of PF6/siRNA nanoparticles. We identified that PF6/siRNA treatments were not associated with any *in vitro* toxicity, as measured by Wst-1 assay, while lipofection and Tat-DRBD had an adverse effects on the cell viability in some cases (i.e in HUVEC cells). Additionally, genome wide microarray analysis was carried out to see if these treatments were associated with vector/siRNA-associated off-target effects. PF6/siRNA treatments affected significantly less transcripts than lipofection at the mRNA levels and these effects were also corroborated at the protein levels, where lipofection altered the expression of twice as many proteins as PF6/siRNA nanoparticles.

The fact that PF6/siRNA nanoparticles induced significant gene silencing in cell cultures without any toxic or immunological side-effects encouraged us to evaluate the full potential of this platform for systemic delivery of siRNA *in vivo*. We chose to target the same HPRT1 mRNA, which we had successfully downregulated *in vitro*. We administered PF6/HPRT1-siRNA nanoparticles intravenously at an siRNA dose of 1 mg/kg and these treatments resulted in efficient knockdown of HPRT1 mRNA in a variety of organs, including liver, kidney and lung. While there are many siRNA delivery platforms that are able to induce gene silencing in liver, targeting other organs is only very seldom achieved. Strikingly, we observed >60% of silencing also in kidneys and lungs. Also, treatments with PF6/siRNA nanoparticles were not associated with any systemic toxicity or immunological side effects.

Based on the strong RNAi responses in the liver, we opted to use another *in vivo* model for liver-specific silencing. In this model, mice were stably expressing luciferase from liver and were treated with PF6/luc-siRNA nanoparticles at various doses over a long period of time. At a dose of 1 mg/kg of siRNA, substantial knockdown of luciferase was achieved and these effects lasted for nearly two weeks. Moreover, efficient gene silencing was seen at a dose of 0.2 mg/kg. Interestingly, these results were at least in line with the effects of the so-called golden standard technique for liver transduction – hydrodynamic injection [195].

These results on siRNA delivery to the liver are comparable to the recently published results with lipid nanoparticles (LNPs) [196] and lipidoids [197]. However, in these reports these vehicles were additionally modified with PEGs and/or cholesterol. Lately, gene silencing in liver has been reported at even lower doses with lipidoids [198] and stable nucleic acid-lipid particles (SNALPs) [199]. Unfortunately, these multicomponent systems require extremely cumbersome formulation procedures, PEGylation and/or cholesterol moieties in order to be active, while PF6 works as a single component vector. Also, in this study we used unmodified siRNAs, whereas in these other reports,

siRNAs have been modified to be more stable in the systemic circulation. Therefore, there is no doubt that PF6 is a very efficient siRNA delivery vehicle *in vivo*, while there is much room for improvement, and by using stabilized siRNAs in combination with optimized formulations, the PF6/siRNA nanoparticles are likely to work at significantly lower doses.

Conclusively, PF6 is a new potent vector for siRNA transfections *in vitro*, as it is efficient in primary and suspension cells, where most of the other vectors are not efficient enough. More importantly, PF6 is very efficient for systemic delivery of siRNAs *in vivo*, with the delivery efficiency in a similar range as with the most potent delivery vectors available. Therefore, it would be extremely interesting to see the real potential of this system in a disease-relevant model.

4.4. PepFect14-mediated delivery of SCOs

We recently reported that the stearyl-TP10 vector can mediate delivery of SCOs upon non-covalent vectorization [131]. We sought to investigate if modifications in the TP10 peptide backbone, by using ornithines instead of lysines, would increase the stability, particle formation efficiency and serum stability of stearyl-TP10 peptide and to study if this new PF14 peptide has enhanced SCO delivery properties.

First of all, we screened if PF14/SCO nanoparticles would be able to confer splice-correction activity in the abovementioned HeLa pLuc 705 cell line [187]. Interestingly, these particles induced splice correction levels, which significantly exceeded the delivery efficiency of regularly used delivery vehicles, such as LF2000, Oligofectamine, RNAiMAX and jetPEI. Moreover, PF14 retained most of its delivery efficiency also in the presence of serum. The difference in delivery efficiency as compared to stearyl-TP10, clearly show that introduction of ornithines increases the stability of this peptide that increases the activity substantially. At the mRNA level, PF14/SCO particles had EC_{50} values of around 100 nM, which is significantly lower than any of the most efficient CPP-platform used for the SCO delivery.

After evaluating the splice correction efficiency in a HeLa pLuc705 assay, we choose to target splicing in a disease-relevant cell model. For this we used H2K mdx mice myotubes, a leading model for Duchenne muscular dystrophy (DMD). In this DMD model, PF14/SCO nanoparticles induced significant splice correction at substantially low concentration (of around 250 nM). That is an extremely encouraging result, as most efficient peptide-based strategies reach the same efficiency at micromolar concentrations.

Additionally, we showed how PF14/SCO nanoparticles could be dried and incorporated into solid dispersions. After the reconstitution of these formulations, particles retained their activity and induced significant splice correction, even after being stored at elevated temperatures for several months. To our

knowledge, this was the first time when CPP-based nanoparticles have been formulated in this manner.

Conclusively, PF14 is an extremely interesting delivery vector for non-covalent vectorization of SCOs and is superior to any other delivery vehicle available at the moment for SCOs. Importantly, it has shown its delivery potential also in disease-relevant DMD model. As a result, PF14 is a delivery vectors that requires immediate evaluation for *in vivo* delivery and could have significant potential in pre-clinical and clinical studies for treatment of DMD.

5. CONCLUSIONS

The key findings of these 4 papers are described below.

- **Paper I.** In this work we modified the $(RxR)_4$ peptide with stearic acid and showed that this stearyl-$(RxR)_4$ vector can be utilized in a non-covalent nanoparticle formation approach. Stearyl-$(RxR)_4$ mediated efficient *in vitro* delivery of both pDNA and SCOs, inducing increased gene expression and correction of splicing, respectively.

- **Paper II.** In this work we aimed to extrapolate the SCO-delivery properties of stearyl-TP10 vector for the delivery of pDNA. Stearyl-TP10 efficiently formed nanoparticles with pDNA and induced efficient gene delivery in a variety of cell lines, including primary cells. Moreover, these nanoparticles mediated efficient gene transfer *in vivo* upon local delivery to the skin and muscle.

- **Paper III.** In this paper, we modified the TP10 vector to be compatible with the cytoplasmic delivery of siRNAs while keeping the frame of stearyl-TP10 peptide. This vector formed stable nanoparticles with siRNA and induced gene silencing in a variety of reporter cell lines. Moreover, by targeting endogenous mRNA, significant gene silencing was achieved in "difficult-to-transfect" cells, including mES cells. Strikingly, by using two *in vivo* models, it was demonstrated that, upon systemic administration of these nanoparticles, efficient gene silencing was achieved in different organs, including liver, kidneys and lungs.

- **Paper IV.** In this paper we aimed to make amino acid substitutions in the stearyl-TP10 backbone to increase both the nanoparticle formation efficiency and enhance the stability of the peptide. PF14 peptide efficiently formed nanoparticles and facilitated the delivery of SCOs, concomitantly resulting in significant splice correction in two *in vitro* models, including in a disease-relevant DMD model. Finally, PF14/SCO particles retained their activity after being formulated as solid dispersions.

Conclusively, the vectors presented in this thesis form a platform of non-viral delivery vehicles that could be utilized for the delivery of various ON-based molecules, i.e. pDNA, SCOs and siRNAs, for gene therapy or gene expression modulation approaches. While these vectors can act as efficient transfection agents *in vitro*, these vectors also possess significant delivery potential *in vivo*, as shown in case of stearyl-TP10 for local delivery of pDNA and in case of PF6 for systemic delivery of siRNAs. Importantly, these vectors do not evoke toxic side-effects, as confirmed by simple toxicity evaluations carried out for all of the vectors. Similarly, *in vivo* treatments with these nanoparticles did not cause

any toxic or immunological side effects or affect common clinical chemistry parameters. This means that these vectors require more thorough *in vivo* investigation, as they clearly show potential to be used in pre-clinical and clinical studies in the future.

SUMMARY IN ESTONIAN

Nukleiinhapete transport rakku sisenevate peptiididega geeniekspressiooni moduleerimise eesmärgil

Viimaste aastakümnete teaduslike saavutuste abil on loodud suur hulk erinevaid meetodeid, mis võimaldavad reguleerida geeniekspressiooni. Enamik geeniekspressiooni mõjutamiseks kasutatavaid molekule põhinevad nukleiinhapetel (NH-del) ja nende analoogidel, varieerudes lühikestest oligonukleotiididest (ON) kuni suurte plasmiidse DNA (pDNA) molekulideni. Oma füsikokeemilistest omadustest tingituna ei ole need molekulid ise võimelised bioloogilisi membraane läbima ning oma toimekohtadeni jõudmiseks vajavad nad transportvektorite (vektorite) abi. Viiruslikud vektorid on väga efektiivsed genoomse materjali rakkudesse viimiseks, kuid nende kasutamine on seotud raskete kõrvaltoimetega, põhjustades näiteks tugevaid immunoloogilisi reaktsioone ja vereloome kasvajaid. Samuti ei ole viiruslikud vektorid sobilikud lühikeste oligonukleotiidide transportimiseks. Seetõttu pööratakse aina rohkem tähelepanu mitteviiruslike vektorite väljaarendamisele.

Rakku sisenevad peptiidid (RSP-d) on üks grupp mitteviiruslikke vektoreid, mis on leidnud laialdast kasutust alates nende avastamisest 1994. a. RSP-d on katioonsed ja/või amfipaatsed peptiidid, mis koosnevad 5–40 aminohappest ning on võimelised rakkudesse transportima erinevaid bioloogiliselt aktiivseid kargomolekule, nende hulgas pDNA-d, splaissingut korrigeerivaid ON-e (SKO) ja väikeseid interfereeruvaid RNA-sid (siRNA), seda nii *in vitro* kui *in vivo* tingimustes. Kargomolekule saab RSP-dega ühendada kahel meetodil, kas kovalentse sidemega või mittekovalentselt nanopartikliteid moodustades. On teada, et RSP-d kasutavad rakkudesse sisenemisel valdavalt erinevaid endotsütoosiradasid ning seetõttu jäävad RSP-d tihti endotsütootilistesse vesiikulitesse kinni. See limiteerib RSP-de ja nendega seotud kargomolekulide biosaadavust, kuna viimaste sihtmärgid asetsevad põhiliselt tsütoplasmas ja rakutuumas. Seetõttu tuleb efektiivsete RSP-de loomiseks neid modifitseerida, et suurendada nende võimet koos kargomolekuliga endosoomist väljuda.

Käesoleva töö eesmärk oli disainida ja iseloomustada uusi RSP-del põhinevaid vektoreid, mis moodustaksid mitte-kovalentselt nanopartikleid erinevate NH-del põhinevate molekulidega ning transpordiksid neid molekule, seal hulgas pDNA-d geenide transportimiseks, SKO-sid splaissingu moduleerimiseks ning siRNA-sid geeniekpressiooni vaigistamiseks, nii *in vitro* kui *in vivo* tingimustes. Modifikatsioonidena lisasime RSP-dele stearhappe ja/või uudse endosomotroopse modifikatsiooni või muutsime RSP aminohappelist järjestust.

Esiteks lisasime prekliinilises kasutuses olevale $(RxR)_4$ peptiidile stearhappe (stearüül-$(RxR)_4$). Saadud vektor moodustas nanopartikleid NH-dega ning võimaldas nende sisenemist rakkudesse. Me näitasime, et stearüül-$(RxR)_4$ transportis pDNA-d erinevatesse rakuliinidesse ning suurendas oluliselt reportergeeni ekspressiooni taset. Stearüül-$(RxR)_4$ oli efektiivne ka SKO-de transpordil,

suurendades splaissingu korrigeerimise taset kuni 20 korda. Võrreldes $(RxR)_4$-PMO kovalentse konjugaadiga, mida kasutatakse prekliinilistes uuringutes ning mis on hetkel üks efektiivsemaid SKO-de vektoreid, saavutati sama splaissingu korrigeerimise tase 10 korda madalamal kontsentratsioonil.

Teises töös uurisime, kas stearüül-TP10 RSP, mille puhul on varem näidatud, et ta on võimeline vektoriseerima SKO-sid mitte-kovalentselt, võiks olla kasutatav ka oluliselt suuremate pDNA-de transpordiks. Stearüül-TP10-ga moodustatud nanopartiklid transportisid pDNA-d efektiivselt mitmetesse rakuliinidesse, sealhulgas ka raskesti transfekteeritavatesse primaarsetesse rakkudesse. Need tulemused julgustasid meid proovima seda süsteemi *in vivo* tingimustes. Pärast intramuskulaarset ja intradermaalset manustamist suurenes reportergeeni ekspressioon võrreldes kontrolltasemega umbes 10 korda, mis on võrdväärne samadel eesmärkidel kasutatavate praegu teadaolevalt kõige tõhusamate vektoritega.

Kolmandas töös disainisime uue endosomotroopse modifikatsiooni, mis võimaldaks vektori kiiret vabanemist endosoomidest ning oleks seetõttu ühilduv siRNA-de tsütoplasmaatilise transpordiga. Me lisasime selle modifikatsiooni stearüül-TP10 peptiidile, luues nii PepFect6 peptiidi. PepFect6/siRNA nanopartiklid vaigistasid väga efektiivselt geeniekspressiooni mitmetes reporterrakuliinides. Seejärel näitasime, et antud süsteem võimaldab efektiivset geeniekpressiooni vaigistamist ka mitmetes väga raskesti transfekteeritavates primaarsetes ja suspensiooni rakkudes ning rakuliinides, k.a. hiire tüvirakkudes. PepFect6 efektiivsuseni *in vitro* tasemel ei küündinud ükski võrdluseks kasutatav kommertsiaalne vektor. Lõpuks näitasime kahte erinevat *in vivo* mudelit kasutades, kuidas pärast nanopartiklite süsteemset manustamist võimaldas nende koosseisus olev siRNA geeniekspressiooni vaigistamist mitmetes organites, k.a. maksas, kopsudes ja neerudes. PepFect6 efektiivsus küündis tasemele, mis oli samaväärne efektiivseimate siRNA transpordivektoritega süsteemseks kasutamiseks.

Neljandas töös tegime muudatusi stearüül-TP10 aminohappelises järjestuses, et tõsta selle bioloogilist stabiilsust ja muuta nanopartiklite moodustamiseks sobivamaks, pannes aluse PepFect14 peptiidile. PepFect14 moodustas SKO-dega väga stabiilseid nanopartikleid, mis võimaldasid efektiivset SKO transporti rakutuuma ja suurendasid splaissingu korrigeerimise taset mitusada korda. Veel enam, kasutades Duchenne´i muskulaarse düstroofia *in vitro* mudelit, näitasime, et PepFect14 võimaldab splaissingu korrigeerimist ka haigusmudelis. Viimaks me näitasime, kuidas PepFect14/SCO nanopartikleid sai moodustada tahkete dispersioonidena, mis pärast uuesti lahustamist säilitasid oma transportomadused. See on oluline leid farmatseutilis-tehnoloogilisest aspektist.

Kokkuvõttes moodustavad kirjeldatud peptiidid mitteviiruslike transportvekotrite platvormi erinevate NH-del ja nende analoogidel põhinevate molekulide transpordiks, mida saab kasutada geeniteraapia eesmärkidel ning geeniekpressiooni moduleerimisel. Lisaks sellele, et nad on efektiivsed *in vitro* transfektsioonivahendid, omavad need peptiidid potentsiaali ka NH-de *in vivo*

transpordis, nagu näidatud stearüül-TP10 puhul paikselt ning PepFect6 puhul süsteemsel manustamisel. Oluline on ka see, et nende kasutamine ei ole seotud toksiliste kõrvaltoimetega, kuna *in vitro* uuringud kinnitasid kõikide vektorite puhul toksilisuse puudumist. Samuti *in vivo* kasutamisel ei täheldatud toksilisi või immunogeenseid kõrvalnähte ega leitud muutusi kliinilises verepildis. Seega antud töös kirjeldatud vektorid vajavad põhjalikke *in vivo* uuringuid, sest nad kahtlemata omavad potentsiaali kasutamiseks prekliinilistes ja kliinilistes uuringutes.

REFERENCES

1. Derossi, D, Joliot, AH, Chassaing, G, and Prochiantz, A (1994). The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269**: 10444–10450.
2. Mulligan, RC (1993). The basic science of gene therapy. *Science* **260**: 926–932.
3. Lander, ES, Linton, LM, Birren, B, Nusbaum, C, Zody, MC, Baldwin, J, Devon, K, Dewar, K, Doyle, M, FitzHugh, W, Funke, R, Gage, D, Harris, K, Heaford, A, Howland, J, Kann, L, Lehoczky, J, LeVine, R, McEwan, P, McKernan, K, Meldrim, J, Mesirov, JP, Miranda, C, Morris, W, Naylor, J, Raymond, C, Rosetti, M, Santos, R, Sheridan, A, Sougnez, C, Stange-Thomann, N, Stojanovic, N, Subramanian, A, Wyman, D, Rogers, J, Sulston, J, Ainscough, R, Beck, S, Bentley, D, Burton, J, Clee, C, Carter, N, Coulson, A, Deadman, R, Deloukas, P, Dunham, A, Dunham, I, Durbin, R, French, L, Grafham, D, Gregory, S, Hubbard, T, Humphray, S, Hunt, A, Jones, M, Lloyd, C, McMurray, A, Matthews, L, Mercer, S, Milne, S, Mullikin, JC, Mungall, A, Plumb, R, Ross, M, Shownkeen, R, Sims, S, Waterston, RH, Wilson, RK, Hillier, LW, McPherson, JD, Marra, MA, Mardis, ER, Fulton, LA, Chinwalla, AT, Pepin, KH, Gish, WR, Chissoe, SL, Wendl, MC, Delehaunty, KD, Miner, TL, Delehaunty, A, Kramer, JB, Cook, LL, Fulton, RS, Johnson, DL, Minx, PJ, Clifton, SW, Hawkins, T, Branscomb, E, Predki, P, Richardson, P, Wenning, S, Slezak, T, Doggett, N, Cheng, JF, Olsen, A, Lucas, S, Elkin, C, Uberbacher, E, Frazier, M, Gibbs, RA, Muzny, DM, Scherer, SE, Bouck, JB, Sodergren, EJ, Worley, KC, Rives, CM, Gorrell, JH, Metzker, ML, Naylor, SL, Kucherlapati, RS, Nelson, DL, Weinstock, GM, Sakaki, Y, Fujiyama, A, Hattori, M, Yada, T, Toyoda, A, Itoh, T, Kawagoe, C, Watanabe, H, Totoki, Y, Taylor, T, Weissenbach, J, Heilig, R, Saurin, W, Artiguenave, F, Brottier, P, Bruls, T, Pelletier, E, Robert, C, Wincker, P, Smith, DR, Doucette-Stamm, L, Rubenfield, M, Weinstock, K, Lee, HM, Dubois, J, Rosenthal, A, Platzer, M, Nyakatura, G, Taudien, S, Rump, A, Yang, H, Yu, J, Wang, J, Huang, G, Gu, J, Hood, L, Rowen, L, Madan, A, Qin, S, Davis, RW, Federspiel, NA, Abola, AP, Proctor, MJ, Myers, RM, Schmutz, J, Dickson, M, Grimwood, J, Cox, DR, Olson, MV, Kaul, R, Shimizu, N, Kawasaki, K, Minoshima, S, Evans, GA, Athanasiou, M, Schultz, R, Roe, BA, Chen, F, Pan, H, Ramser, J, Lehrach, H, Reinhardt, R, McCombie, WR, de la Bastide, M, Dedhia, N, Blocker, H, Hornischer, K, Nordsiek, G, Agarwala, R, Aravind, L, Bailey, JA, Bateman, A, Batzoglou, S, Birney, E, Bork, P, Brown, DG, Burge, CB, Cerutti, L, Chen, HC, Church, D, Clamp, M, Copley, RR, Doerks, T, Eddy, SR, Eichler, EE, Furey, TS, Galagan, J, Gilbert, JG, Harmon, C, Hayashizaki, Y, Haussler, D, Hermjakob, H, Hokamp, K, Jang, W, Johnson, LS, Jones, TA, Kasif, S, Kaspryzk, A, Kennedy, S, Kent, WJ, Kitts, P, Koonin, EV, Korf, I, Kulp, D, Lancet, D, Lowe, TM, McLysaght, A, Mikkelsen, T, Moran, JV, Mulder, N, Pollara, VJ, Ponting, CP, Schuler, G, Schultz, J, Slater, G, Smit, AF, Stupka, E, Szustakowski, J, Thierry-Mieg, D, Thierry-Mieg, J, Wagner, L, Wallis, J, Wheeler, R, Williams, A, Wolf, YI, Wolfe, KH, Yang, SP, Yeh, RF, Collins, F, Guyer, MS, Peterson, J, Felsenfeld, A, Wetterstrand, KA, Patrinos, A, Morgan, MJ, de Jong, P, Catanese, JJ, Osoegawa, K, Shizuya, H, Choi, S, and Chen, YJ (2001). Initial sequencing and analysis of the human genome. *Nature* **409**: 860–921.

4. Lander, ES (2011). Initial impact of the sequencing of the human genome. *Nature* **470**: 187–197.
5. Walsh, CE (2003). Gene therapy progress and prospects: gene therapy for the hemophilias. *Gene Ther* **10**: 999–1003.
6. Trollet, C, Athanasopoulos, T, Popplewell, L, Malerba, A, and Dickson, G (2009). Gene therapy for muscular dystrophy: current progress and future prospects. *Expert Opin Biol Ther* **9**: 849–866.
7. Griesenbach, U, Geddes, DM, and Alton, EW (2004). Advances in cystic fibrosis gene therapy. *Curr Opin Pulm Med* **10**: 542–546.
8. Gaffney, MM, Hynes, SO, Barry, F, and O'Brien, T (2007). Cardiovascular gene therapy: current status and therapeutic potential. *Br J Pharmacol* **152**: 175–188.
9. Rissanen, TT, and Yla-Herttuala, S (2007). Current status of cardiovascular gene therapy. *Mol Ther* **15**: 1233–1247.
10. Burton, EA, Glorioso, JC, and Fink, DJ (2003). Gene therapy progress and prospects: Parkinson's disease. *Gene Ther* **10**: 1721–1727.
11. Samaranayake, H, Maatta, AM, Pikkarainen, J, and Yla-Herttuala, S (2010). Future prospects and challenges of antiangiogenic cancer gene therapy. *Hum Gene Ther* **21**: 381–396.
12. Kerr, D (2003). Clinical development of gene therapy for colorectal cancer. *Nat Rev Cancer* **3**: 615–622.
13. Thomas, CE, Ehrhardt, A, and Kay, MA (2003). Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet* **4**: 346–358.
14. Kay, MA, Glorioso, JC, and Naldini, L (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* **7**: 33–40.
15. Cavazzana-Calvo, M, Hacein-Bey, S, de Saint Basile, G, Gross, F, Yvon, E, Nusbaum, P, Selz, F, Hue, C, Certain, S, Casanova, JL, Bousso, P, Deist, FL, and Fischer, A (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**: 669–672.
16. Kay, MA, Manno, CS, Ragni, MV, Larson, PJ, Couto, LB, McClelland, A, Glader, B, Chew, AJ, Tai, SJ, Herzog, RW, Arruda, V, Johnson, F, Scallan, C, Skarsgard, E, Flake, AW, and High, KA (2000). Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* **24**: 257–261.
17. Hacein-Bey-Abina, S, Von Kalle, C, Schmidt, M, McCcormack, MP, Wulffraat, N, Leboulch, P, Lim, A, Osborne, CS, Pawliuk, R, Morillon, E, Sorensen, R, Forster, A, Fraser, P, Cohen, JI, de Saint Basile, G, Alexander, I, Wintergerst, U, Frebourg, T, Aurias, A, Stoppa-Lyonnet, D, Romana, S, Radford-Weiss, I, Gross, F, Valensi, F, Delabesse, E, Macintyre, E, Sigaux, F, Soulier, J, Leiva, LE, Wissler, M, Prinz, C, Rabbitts, TH, Le Deist, F, Fischer, A, and Cavazzana-Calvo, M (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–419.
18. Crooke, ST (2004). Progress in antisense technology. *Annu Rev Med* **55**: 61–95.
19. Opalinska, JB, and Gewirtz, AM (2002). Nucleic-acid therapeutics: basic principles and recent applications. *Nat Rev Drug Discov* **1**: 503–514.
20. Cutrona, G, Carpaneto, EM, Ulivi, M, Roncella, S, Landt, O, Ferrarini, M, and Boffa, LC (2000). Effects in live cells of a c-myc anti-gene PNA linked to a nuclear localization signal. *Nat Biotechnol* **18**: 300–303.

21. Janowski, BA, Kaihatsu, K, Huffman, KE, Schwartz, JC, Ram, R, Hardy, D, Mendelson, CR, and Corey, DR (2005). Inhibiting transcription of chromosomal DNA with antigene peptide nucleic acids. *Nat Chem Biol* **1**: 210–215.
22. Gleave, ME, and Monia, BP (2005). Antisense therapy for cancer. *Nat Rev Cancer* **5**: 468–479.
23. Morris, KV (2009). RNA-directed transcriptional gene silencing and activation in human cells. *Oligonucleotides* **19**: 299–306.
24. Bennett, CF, and Swayze, EE (2010). RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu Rev Pharmacol Toxicol* **50**: 259–293.
25. Paterson, BM, Roberts, BE, and Kuff, EL (1977). Structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. *Proc Natl Acad Sci U S A* **74**: 4370–4374.
26. Stephenson, ML, and Zamecnik, PC (1978). Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci U S A* **75**: 285–288.
27. Zamecnik, PC, and Stephenson, ML (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci U S A* **75**: 280–284.
28. Matlin, AJ, Clark, F, and Smith, CW (2005). Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol* **6**: 386–398.
29. Black, DL (2003). Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem* **72**: 291–336.
30. Garcia-Blanco, MA, Baraniak, AP, and Lasda, EL (2004). Alternative splicing in disease and therapy. *Nat Biotechnol* **22**: 535–546.
31. Caceres, JF, and Kornblihtt, AR (2002). Alternative splicing: multiple control mechanisms and involvement in human disease. *Trends Genet* **18**: 186–193.
32. Pajares, MJ, Ezponda, T, Catena, R, Calvo, A, Pio, R, and Montuenga, LM (2007). Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncol* **8**: 349–357.
33. Bauman, J, Jearawiriyapaisarn, N, and Kole, R (2009). Therapeutic potential of splice-switching oligonucleotides. *Oligonucleotides* **19**: 1–13.
34. Dominski, Z, and Kole, R (1993). Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc Natl Acad Sci U S A* **90**: 8673–8677.
35. Lacerra, G, Sierakowska, H, Carestia, C, Fucharoen, S, Summerton, J, Weller, D, and Kole, R (2000). Restoration of hemoglobin A synthesis in erythroid cells from peripheral blood of thalassemic patients. *Proc Natl Acad Sci U S A* **97**: 9591–9596.
36. Sierakowska, H, Sambade, MJ, Agrawal, S, and Kole, R (1996). Repair of thalassemic human beta-globin mRNA in mammalian cells by antisense oligonucleotides. *Proc Natl Acad Sci U S A* **93**: 12840–12844.
37. Kole, R, Williams, T, and Cohen, L (2004). RNA modulation, repair and remodeling by splice switching oligonucleotides. *Acta Biochim Pol* **51**: 373–378.
38. Koenig, M, Beggs, AH, Moyer, M, Scherpf, S, Heindrich, K, Bettecken, T, Meng, G, Muller, CR, Lindlof, M, Kaariainen, H, and et al. (1989). The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *Am J Hum Genet* **45**: 498–506.
39. Wood, MJ, Gait, MJ, and Yin, H (2010). RNA-targeted splice-correction therapy for neuromuscular disease. *Brain* **133**: 957–972.

40. Aartsma-Rus, A, Fokkema, I, Verschuuren, J, Ginjaar, I, van Deutekom, J, van Ommen, GJ, and den Dunnen, JT (2009). Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum Mutat* **30**: 293–299.
41. Aartsma-Rus, A (2010). Antisense-mediated modulation of splicing: therapeutic implications for Duchenne muscular dystrophy. *RNA Biol* **7**: 453–461.
42. Aartsma-Rus, A, and van Ommen, GJ (2010). Progress in therapeutic antisense applications for neuromuscular disorders. *Eur J Hum Genet* **18**: 146–153.
43. Koshkin, AA, and Wengel, J (1998). Synthesis of Novel 2',3'-Linked Bicyclic Thymine Ribonucleosides. *J Org Chem* **63**: 2778–2781.
44. Morvan, F, Porumb, H, Degols, G, Lefebvre, I, Pompon, A, Sproat, BS, Rayner, B, Malvy, C, Lebleu, B, and Imbach, JL (1993). Comparative evaluation of seven oligonucleotide analogues as potential antisense agents. *J Med Chem* **36**: 280–287.
45. Baker, BF, Lot, SS, Condon, TP, Cheng-Flournoy, S, Lesnik, EA, Sasmor, HM, and Bennett, CF (1997). 2'-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. *J Biol Chem* **272**: 11994–12000.
46. Summerton, J, and Weller, D (1997). Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev* **7**: 187–195.
47. Egholm, M, Buchardt, O, Christensen, L, Behrens, C, Freier, SM, Driver, DA, Berg, RH, Kim, SK, Norden, B, and Nielsen, PE (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* **365**: 566–568.
48. Bernstein, E, Caudy, AA, Hammond, SM, and Hannon, GJ (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**: 363–366.
49. Hammond, SM, Bernstein, E, Beach, D, and Hannon, GJ (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404**: 293–296.
50. Rand, TA, Ginalski, K, Grishin, NV, and Wang, X (2004). Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. *Proc Natl Acad Sci U S A* **101**: 14385–14389.
51. Matranga, C, Tomari, Y, Shin, C, Bartel, DP, and Zamore, PD (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**: 607–620.
52. Hutvagner, G, and Zamore, PD (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**: 2056–2060.
53. Bartlett, DW, and Davis, ME (2006). Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res* **34**: 322–333.
54. Fire, A, Xu, S, Montgomery, MK, Kostas, SA, Driver, SE, and Mello, CC (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**: 806–811.
55. Elbashir, SM, Harborth, J, Lendeckel, W, Yalcin, A, Weber, K, and Tuschl, T (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 494–498.

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56. Song, E, Lee, SK, Wang, J, Ince, N, Ouyang, N, Min, J, Chen, J, Shankar, P, and Lieberman, J (2003). RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med* **9**: 347–351.
57. Kim, DH, and Rossi, JJ (2007). Strategies for silencing human disease using RNA interference. *Nat Rev Genet* **8**: 173–184.
58. Vaishnaw, AK, Gollob, J, Gamba-Vitalo, C, Hutabarat, R, Sah, D, Meyers, R, de Fougerolles, T, and Maraganore, J (2010). A status report on RNAi therapeutics. *Silence* **1**: 14.
59. Castanotto, D, and Rossi, JJ (2009). The promises and pitfalls of RNA-interference-based therapeutics. *Nature* **457**: 426–433.
60. Whitehead, KA, Langer, R, and Anderson, DG (2009). Knocking down barriers: advances in siRNA delivery. *Nat Rev Drug Discov* **8**: 129–138.
61. de Fougerolles, A, Vornlocher, HP, Maraganore, J, and Lieberman, J (2007). Interfering with disease: a progress report on siRNA-based therapeutics. *Nat Rev Drug Discov* **6**: 443–453.
62. Glover, DJ, Lipps, HJ, and Jans, DA (2005). Towards safe, non-viral therapeutic gene expression in humans. *Nat Rev Genet* **6**: 299–U229.
63. Pack, DW, Hoffman, AS, Pun, S, and Stayton, PS (2005). Design and development of polymers for gene delivery. *Nat Rev Drug Discov* **4**: 581–593.
64. Schaffert, D, and Wagner, E (2008). Gene therapy progress and prospects: synthetic polymer-based systems. *Gene Ther* **15**: 1131–1138.
65. Viola, JR, El-Andaloussi, S, Oprea, II, and Smith, CIE (2010). Non-viral nanovectors for gene delivery: factors that govern successful therapeutics. *Expert Opin Drug Deliv* **7**: 721–735.
66. Thierry, AR, Abes, S, Resina, S, Travo, A, Richard, JP, Prevot, P, and Lebleu, B (2006). Comparison of basic peptides- and lipid-based strategies for the delivery of splice correcting oligonucleotides. *Biochim Biophys Acta* **1758**: 364–374.
67. Felgner, PL, Gadek, TR, Holm, M, Roman, R, Chan, HW, Wenz, M, Northrop, JP, Ringold, GM, and Danielsen, M (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* **84**: 7413–7417.
68. Scheule, RK, St George, JA, Bagley, RG, Marshall, J, Kaplan, JM, Akita, GY, Wang, KX, Lee, ER, Harris, DJ, Jiang, C, Yew, NS, Smith, AE, and Cheng, SH (1997). Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Hum Gene Ther* **8**: 689–707.
69. Filion, MC, and Phillips, NC (1998). Major limitations in the use of cationic liposomes for DNA delivery. *Int J Pharm* **162**: 159–170.
70. Green, M, and Loewenstein, PM (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* **55**: 1179–1188.
71. Frankel, AD, and Pabo, CO (1988). Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55**: 1189–1193.
72. Joliot, AH, Triller, A, Volovitch, M, Pernelle, C, and Prochiantz, A (1991). alpha-2,8-Polysialic acid is the neuronal surface receptor of antennapedia homeobox peptide. *New Biol* **3**: 1121–1134.
73. Lindgren, M, and Langel, Ü (2011). Classes and prediction of cell-penetrating peptides. *Methods Mol Biol* **683**: 3–19.
74. Fonseca, SB, Pereira, MP, and Kelley, SO (2009). Recent advances in the use of cell-penetrating peptides for medical and biological applications. *Adv Drug Deliv Rev* **61**: 953–964.

75. Brasseur, R, and Divita, G (2010). Happy birthday cell penetrating peptides: already 20 years. *Biochim Biophys Acta* **1798**: 2177–2181.
76. Hassane, FS, Saleh, AF, Abes, R, Gait, MJ, and Lebleu, B (2010). Cell penetrating peptides: overview and applications to the delivery of oligonucleotides. *Cell Mol Life Sci* **67**: 715–726.
77. Heitz, F, Morris, MC, and Divita, G (2009). Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br J Pharmacol* **157**: 195–206.
78. Juliano, R, Alam, R, Dixit, V, and Kang, HM (2009). Cell-targeting and cell-penetrating peptides for delivery of therapeutic and imaging agents. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **1**: 324–335.
79. Mäe, M, and Langel, Ü (2006). Cell-penetrating peptides as vectors for peptide, protein and oligonucleotide delivery. *Curr Opin Pharmacol* **6**: 509–514.
80. EL Andaloussi, S, Holm, T, and Langel, Ü (2005). Cell-penetrating peptides: mechanisms and applications. *Curr Pharm Des* **11**: 3597–3611.
81. Vives, E, Brodin, P, and Lebleu, B (1997). 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.
82. Pooga, M, Hällbrink, M, Zorko, M, and Langel, Ü (1998). Cell penetration by transportan. *Faseb J* **12**: 67–77.
83. Morris, MC, Vidal, P, Chaloin, L, Heitz, F, and Divita, G (1997). A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res* **25**: 2730–2736.
84. Oehlke, J, Scheller, A, Wiesner, B, Krause, E, Beyermann, M, Klauschenz, E, Melzig, M, and Bienert, M (1998). 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.
85. Futaki, S, Suzuki, T, Ohashi, W, Yagami, T, Tanaka, S, Ueda, K, and Sugiura, Y (2001). Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* **276**: 5836–5840.
86. Ziegler, A (2008). Thermodynamic studies and binding mechanisms of cell-penetrating peptides with lipids and glycosaminoglycans. *Adv Drug Deliv Rev* **60**: 580–597.
87. Lamaziere, A, Burlina, F, Wolf, C, Chassaing, G, Trugnan, G, and Ayala-San-martin, J (2007). Non-metabolic membrane tubulation and permeability induced by bioactive peptides. *PLoS ONE* **2**: e201.
88. Magzoub, M, and Gräslund, A (2004). Cell-penetrating peptides: [corrected] from inception to application. *Q Rev Biophys* **37**: 147–195.
89. Deshayes, S, Morris, MC, Divita, G, and Heitz, F (2005). Cell-penetrating peptides: tools for intracellular delivery of therapeutics. *Cell Mol Life Sci* **62**: 1839–1849.
90. Deshayes, S, Gerbal-Chaloin, S, Morris, MC, Aldrian-Herrada, G, Charnet, P, Divita, G, and Heitz, F (2004). On the mechanism of non-endosomial peptide-mediated cellular delivery of nucleic acids. *Biochim Biophys Acta* **1667**: 141–147.
91. Deshayes, S, Plenat, T, Aldrian-Herrada, G, Divita, G, Le Grimellec, C, and Heitz, F (2004). Primary amphipathic cell-penetrating peptides: structural requirements and interactions with model membranes. *Biochemistry* **43**: 7698–7706.

92. Magzoub, M, Kilk, K, Eriksson, LE, Langel, Ü, and Gräslund, A (2001). Interaction and structure induction of cell-penetrating peptides in the presence of phospholipid vesicles. *Biochim Biophys Acta* **1512**: 77–89.
93. Plenat, T, Deshayes, S, Boichot, S, Milhiet, PE, Cole, RB, Heitz, F, and Le Grimellec, C (2004). Interaction of primary amphipathic cell-penetrating peptides with phospholipid-supported monolayers. *Langmuir* **20**: 9255–9261.
94. Soomets, U, Lindgren, M, Gallet, X, Hällbrink, M, Elmquist, A, Balaspiri, L, Zorko, M, Pooga, M, Brasseur, R, and Langel, Ü (2000). Deletion analogues of transportan. *Biochim Biophys Acta* **1467**: 165–176.
95. Morris, MC, Depollier, J, Mery, J, Heitz, F, and Divita, G (2001). A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat Biotechnol* **19**: 1173–1176.
96. Ghibaudi, E, Boscolo, B, Inserra, G, Laurenti, E, Traversa, S, Barbero, L, and Ferrari, RP (2005). The interaction of the cell-penetrating peptide penetratin with heparin, heparansulfates and phospholipid vesicles investigated by ESR spectroscopy. *J Pept Sci* **11**: 401–409.
97. Dathe, M, Schumann, M, Wieprecht, T, Winkler, A, Beyermann, M, Krause, E, Matsuzaki, K, Murase, O, and Bienert, M (1996). Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry* **35**: 12612–12622.
98. Oehlke, J, Krause, E, Wiesner, B, Beyermann, M, and Bienert, M (1997). Extensive cellular uptake into endothelial cells of an amphipathic beta-sheet forming peptide. *FEBS Lett* **415**: 196–199.
99. Crombez, L, Aldrian-Herrada, G, Konate, K, Nguyen, QN, McMaster, GK, Brasseur, R, Heitz, F, and Divita, G (2009). A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells. *Mol Ther* **17**: 95–103.
100. Ziegler, A, Blatter, XL, Seelig, A, and Seelig, J (2003). Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis. *Biochemistry* **42**: 9185–9194.
101. Thoren, PE, Persson, D, Lincoln, P, and Norden, B (2005). Membrane destabilizing properties of cell-penetrating peptides. *Biophys Chem* **114**: 169–179.
102. Avrahami, D, and Shai, Y (2003). Bestowing antifungal and antibacterial activities by lipophilic acid conjugation to D,L-amino acid-containing antimicrobial peptides: a plausible mode of action. *Biochemistry* **42**: 14946–14956.
103. Malina, A, and Shai, Y (2005). Conjugation of fatty acids with different lengths modulates the antibacterial and antifungal activity of a cationic biologically inactive peptide. *Biochem J* **390**: 695–702.
104. Futaki, S, Ohashi, W, Suzuki, T, Niwa, M, Tanaka, S, Ueda, K, Harashima, H, and Sugiura, Y (2001). Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjug Chem* **12**: 1005–1011.
105. Langel, Ü (2002). *Cell-Penetrating Peptides: Processes and Applications*, CRC Press: Boca Raton, FL.
106. Richard, JP, Melikov, K, Vives, E, Ramos, C, Verbeure, B, Gait, MJ, Chernomordik, LV, and Lebleu, B (2003). Cell-penetrating peptides – A reevaluation of the mechanism of cellular uptake. *J Biol Chem* **278**: 585–590.
107. Lundberg, M, and Johansson, M (2001). Is VP22 nuclear homing an artifact? *Nat Biotechnol* **19**: 713–714.

108. Fuchs, SM, and Raines, RT (2004). Pathway for polyarginine entry into mammalian cells. *Biochemistry* **43**: 2438–2444.
109. Nakase, I, Tadokoro, A, Kawabata, N, Takeuchi, T, Katoh, H, Hiramoto, K, Negishi, M, Nomizu, M, Sugiura, Y, and Futaki, S (2007). Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. *Biochemistry* **46**: 492–501.
110. Futaki, S, Nakase, I, Tadokoro, A, Takeuchi, T, and Jones, AT (2007). Arginine-rich peptides and their internalization mechanisms. *Biochem Soc Trans* **35**: 784–787.
111. Lundin, P, Johansson, H, Guterstam, P, Holm, T, Hansen, M, Langel, Ü, and EL Andaloussi, S (2008). Distinct uptake routes of cell-penetrating peptide conjugates. *Bioconjug Chem* **19**: 2535–2542.
112. Deshayes, S, Konate, K, Aldrian, G, Crombez, L, Heitz, F, and Divita, G (2010). Structural polymorphism of non-covalent peptide-based delivery systems: highway to cellular uptake. *Biochim Biophys Acta* **1798**: 2304–2314.
113. Richard, JP, Melikov, K, Brooks, H, Prevot, P, Lebleu, B, and Chernomordik, LV (2005). Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. *J Biol Chem* **280**: 15300–15306.
114. Potocky, TB, Menon, AK, and Gellman, SH (2003). Cytoplasmic and nuclear delivery of a TAT-derived peptide and a beta-peptide after endocytic uptake into HeLa cells. *J Biol Chem* **278**: 50188–50194.
115. Fittipaldi, A, Ferrari, A, Zoppe, M, Arcangeli, C, Pellegrini, V, Beltram, F, and Giacca, M (2003). Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. *J Biol Chem* **278**: 34141–34149.
116. Wadia, JS, Stan, RV, and Dowdy, SF (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* **10**: 310–315.
117. Duchardt, F, Fotin-Mleczek, M, Schwarz, H, Fischer, R, and Brock, R (2007). A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic* **8**: 848–866.
118. Padari, K, Säälik, P, Hansen, M, Koppel, K, Raid, R, Langel, Ü, and Pooga, M (2005). Cell transduction pathways of transportans. *Bioconjug Chem* **16**: 1399–1410.
119. Fretz, MM, Penning, NA, Al-Taei, S, Futaki, S, Takeuchi, T, Nakase, I, Storm, G, and Jones, AT (2007). Temperature-, concentration- and cholesterol-dependent translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34+ leukaemia cells. *Biochem J* **403**: 335–342.
120. Edenhofer, F (2008). Protein transduction revisited: novel insights into the mechanism underlying intracellular delivery of proteins. *Curr Pharm Des* **14**: 3628–3636.
121. Lamaze, C, and Schmid, SL (1995). The emergence of clathrin-independent pinocytic pathways. *Curr Opin Cell Biol* **7**: 573–580.
122. El-Sayed, A, Futaki, S, and Harashima, H (2009). Delivery of Macromolecules Using Arginine-Rich Cell-Penetrating Peptides: Ways to Overcome Endosomal Entrapment. *AAPS J*. **11**: 13–22.
123. Nakase, I, Kobayashi, S, and Futaki, S (2010). Endosome-disruptive peptides for improving cytosolic delivery of bioactive macromolecules. *Biopolymers* **94**: 763–770.

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124. Varkouhi, AK, Scholte, M, Storm, G, and Haisma, HJ (2010). Endosomal escape pathways for delivery of biologicals. *J Control Release*. In press.
125. Endoh, T, and Ohtsuki, T (2009). Cellular siRNA delivery using cell-penetrating peptides modified for endosomal escape. *Adv Drug Deliv Rev* **61**: 704–709.
126. Lundberg, P, EL Andaloussi, S, Sütlü, T, Johansson, H, and Langel, Ü (2007). Delivery of short interfering RNA using endosomolytic cell-penetrating peptides. *FASEB J* **21**: 2664–2671.
127. Lo, SL, and Wang, S (2008). An endosomolytic Tat peptide produced by incorporation of histidine and cysteine residues as a nonviral vector for DNA transfection. *Biomaterials* **29**: 2408–2414.
128. Khalil, IA, Kogure, K, Futaki, S, Hama, S, Akita, H, Ueno, M, Kishida, H, Kudoh, M, Mishina, Y, Kataoka, K, Yamada, M, and Harashima, H (2007). Octaarginine-modified multifunctional envelope-type nanoparticles for gene delivery. *Gene Ther* **14**: 682–689.
129. Kogure, K, Moriguchi, R, Sasaki, K, Ueno, M, Futaki, S, and Harashima, H (2004). Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method. *Journal of controlled release* **98**: 317–323.
130. Khalil, IA, Futaki, S, Niwa, M, Baba, Y, Kaji, N, Kamiya, H, and Harashima, H (2004). Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation. *Gene Ther* **11**: 636–644.
131. Mäe, M, EL Andaloussi, S, Lundin, P, Oskolkov, N, Johansson, HJ, Guterstam, P, and Langel, Ü (2009). A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy. *J Control Release* **134**: 221–227.
132. Nakamura, Y, Kogure, K, Futaki, S, and Harashima, H (2007). Octaarginine-modified multifunctional envelope-type nano device for siRNA. *J Control Release* **119**: 360–367.
133. Tonges, L, Lingor, P, Egle, R, Dietz, GP, Fahr, A, and Bahr, M (2006). Stearylated octaarginine and artificial virus-like particles for transfection of siRNA into primary rat neurons. *RNA* **12**: 1431–1438.
134. Simeoni, F, Morris, MC, Heitz, F, and Divita, G (2003). 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.
135. Kilk, K, EL Andaloussi, S, Jarver, P, Meikas, A, Valkna, A, Bartfai, T, Kogerman, P, Metsis, M, and Langel, Ü (2005). Evaluation of transportan 10 in PEI mediated plasmid delivery assay. *J Control Release* **103**: 511–523.
136. Liu, Z, Li, M, Cui, D, and Fei, J (2005). Macro-branched cell-penetrating peptide design for gene delivery. *J Control Release* **102**: 699–710.
137. Ignatovich, IA, Dizhe, EB, Pavlotskaya, AV, Akifiev, BN, Burov, SV, Orlov, SV, and Perevozchikov, AP (2003). Complexes of plasmid DNA with basic domain 47–57 of the HIV-1 Tat protein are transferred to mammalian cells by endocytosis-mediated pathways. *J Biol Chem* **278**: 42625–42636.
138. Trabulo, S, Mano, M, Faneca, H, Cardoso, AL, Duarte, S, Henriques, A, Paiva, A, Gomes, P, Simoes, S, and de Lima, MC (2008). S4(13)-PV cell penetrating peptide and cationic liposomes act synergistically to mediate intracellular delivery of plasmid DNA. *J Gene Med* **10**: 1210–1222.

139. Fujita, T, Furuhata, M, Hattori, Y, Kawakami, H, Toma, K, and Maitani, Y (2008). High gene delivery in tumor by intratumoral injection of tetraarginine-PEG lipid-coated protamine/DNA. *J Control Release* **129**: 124–127.
140. Wyman, TB, Nicol, F, Zelphati, O, Scaria, PV, Plank, C, and Szoka, FC (1997). Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. *Biochemistry* **36**: 3008–3017.
141. Morris, MC, Chaloin, L, Mery, J, Heitz, F, and Divita, G (1999). A novel potent strategy for gene delivery using a single peptide vector as a carrier. *Nucleic Acids Res* **27**: 3510–3517.
142. Rittner, K, Benavente, A, Bompard-Sorlet, A, Heitz, F, Divita, G, Brasseur, R, and Jacobs, E (2002). New basic membrane-destabilizing peptides for plasmid-based gene delivery in vitro and in vivo. *Mol Ther* **5**: 104–114.
143. Johnson, LN, Cashman, SM, and Kumar-Singh, R (2008). Cell-penetrating peptide for enhanced delivery of nucleic acids and drugs to ocular tissues including retina and cornea. *Mol Ther* **16**: 107–114.
144. Read, SP, Cashman, S, and Kumar-Singh, R (2010). A poly(ethylene) glycolylated peptide for ocular delivery compacts DNA into nanoparticles for gene delivery to post-mitotic tissues in vivo. *J Gene Med* **12**: 86–96.
145. Read, SP, Cashman, SM, and Kumar-Singh, R (2010). POD Nanoparticles Expressing GDNF Provide Structural and Functional Rescue of Light-induced Retinal Degeneration in an Adult Mouse. *Mol Ther*. In press.
146. Lehto, T, Abes, R, Oskolkov, N, Suhorutšenko, J, Copolovici, DM, Mäger, I, Viola, JR, Simonson, OE, Ezzat, K, Guterstam, P, Eriste, E, Smith, CIE, Lebleu, B, EL Andaloussi, S, and Langel, Ü (2010). Delivery of nucleic acids with a stearylated (RxR)(4) peptide using a non-covalent co-incubation strategy. *Journal of Controlled Release* **141**: 42–51.
147. Lehto, T, Simonson, OE, Mäger, I, Ezzat, K, Sork, H, Copolovici, DM, Viola, JR, Zaghloul, EM, Lundin, P, Moreno, PM, Mäe, M, Oskolkov, N, Suhorutšenko, J, Smith, CIE, and EL Andaloussi, S (2011). A Peptide-based Vector for Efficient Gene Transfer In Vitro and In Vivo. *Mol Ther*. In press.
148. Wang, H-Y, Chen, J-X, Sun, Y-X, Deng, J-Z, Li, C, Zhang, X-Z, and Zhuo, R-X (2010). Construction of cell penetrating peptide vectors with N-terminal stearylated nuclear localization signal for targeted delivery of DNA into the cell nuclei. *Journal of controlled release*. In press.
149. Astriab-Fisher, A, Sergueev, D, Fisher, M, Shaw, BR, and Juliano, RL (2002). Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions. *Pharm Res* **19**: 744–754.
150. Moulton, HM, Hase, MC, Smith, KM, and Iversen, PL (2003). HIV Tat peptide enhances cellular delivery of antisense morpholino oligomers. *Antisense Nucleic Acid Drug Dev* **13**: 31–43.
151. Wolf, Y, Pritz, S, Abes, S, Bienert, M, Lebleu, B, and Oehlke, J (2006). Structural requirements for cellular uptake and antisense activity of peptide nucleic acids conjugated with various peptides. *Biochemistry* **45**: 14944–14954.
152. EL Andaloussi, S, Johansson, HJ, Lundberg, P, and Langel Ü (2006). Induction of splice correction by cell-penetrating peptide nucleic acids. *J Gene Med* **8**: 1262–1273.

153. EL Andaloussi, S, Johansson, HJ, Holm, T, and Langel, Ü (2007). A novel cell-penetrating peptide, M918, for efficient delivery of proteins and peptide nucleic acids. *Mol Ther* **15**: 1820–1826.
154. Abes, S, Moulton, HM, Clair, P, Prevot, P, Youngblood, DS, Wu, RP, Iversen, PL, and Lebleu, B (2006). 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.
155. Moulton, HM, Fletcher, S, Neuman, BW, McClorey, G, Stein, DA, Abes, S, Wilton, SD, Buchmeier, MJ, Lebleu, B, and Iversen, PL (2007). Cell-penetrating peptide-morpholino conjugates alter pre-mRNA splicing of DMD (Duchenne muscular dystrophy) and inhibit murine coronavirus replication in vivo. *Biochem Soc Trans* **35**: 826–828.
156. Abes, R, Moulton, HM, Clair, P, Yang, ST, Abes, S, Melikov, K, Prevot, P, Youngblood, DS, Iversen, PL, Chernomordik, LV, and Lebleu, B (2008). Delivery of steric block morpholino oligomers by (R-X-R)4 peptides: structure-activity studies. *Nucleic Acids Res* **36**: 6343–6354.
157. McClorey, G, Moulton, HM, Iversen, PL, Fletcher, S, and Wilton, SD (2006). Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. *Gene Ther* **13**: 1373–1381.
158. Jearawiriyapaisarn, N, Moulton, HM, Buckley, B, Roberts, J, Sazani, P, Fucharoen, S, Iversen, PL, and Kole, R (2008). Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. *Mol Ther* **16**: 1624–1629.
159. Fletcher, S, Honeyman, K, Fall, AM, Harding, PL, Johnsen, RD, Steinhaus, JP, Moulton, HM, Iversen, PL, and Wilton, SD (2007). Morpholino oligomer-mediated exon skipping averts the onset of dystrophic pathology in the mdx mouse. *Mol Ther* **15**: 1587–1592.
160. Burrer, R, Neuman, BW, Ting, JP, Stein, DA, Moulton, HM, Iversen, PL, Kuhn, P, and Buchmeier, MJ (2007). Antiviral effects of antisense morpholino oligomers in murine coronavirus infection models. *J Virol* **81**: 5637–5648.
161. Abes, S, Turner, JJ, Ivanova, GD, Owen, D, Williams, D, Arzumanov, A, Clair, P, Gait, MJ, and Lebleu, B (2007). Efficient splicing correction by PNA conjugation to an R6-Penetratin delivery peptide. *Nucleic Acids Res* **35**: 4495–4502.
162. Ivanova, GD, Arzumanov, A, Abes, R, Yin, H, Wood, MJ, Lebleu, B, and Gait, MJ (2008). Improved cell-penetrating peptide-PNA conjugates for splicing redirection in HeLa cells and exon skipping in mdx mouse muscle. *Nucleic Acids Res* **36**: 6418–28.
163. Yin, H, Moulton, HM, Seow, Y, Boyd, C, Boutilier, J, Iverson, P, and Wood, MJ (2008). Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function. *Hum Mol Genet* **17**: 3909–18.
164. Ezzat, K, EL Andaloussi, S, Zaghloul, EM, Lehto, T, Lindberg, S, Moreno, PM, Viola, JR, Magdy, T, Abdo, R, Guterstam, P, Sillard, R, Hammond, SM, Wood, MJ, Arzumanov, AA, Gait, MJ, Smith, CIE, Hällbrink, M, and Langel, Ü (2011). PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. *Nucleic Acids Res.* In press.
165. Yin, H, Moulton, HM, Betts, C, Merritt, T, Seow, Y, Ashraf, S, Wang, Q, Boutilier, J, and Wood, MJ (2010). Functional rescue of dystrophin-deficient mdx mice by a chimeric peptide-PMO. *Mol Ther* **18**: 1822–1829.

166. Yin, H, Moulton, HM, Betts, C, Seow, Y, Boutilier, J, Iverson, PL, and Wood, MJ (2009). A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient mdx mice. *Hum Mol Genet* **18**: 4405–4414.
167. Svasti, S, Suwanmanee, T, Fucharoen, S, Moulton, H, Nelson, M, Maeda, N, Smithies, O, and Kole, R (2009). RNA repair restores hemoglobin expression in IVS2-654 thalassemic mice. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 1205–1210.
168. EL Andaloussi, S, Lehto, T, Lundin, P, and Langel, Ü (2011). Application of PepFect peptides for the delivery of splice-correcting oligonucleotides. *Methods Mol Biol* **683**: 361–373.
169. Crombez, L, Morris, MC, Dufort, S, Aldrian-Herrada, G, Nguyen, Q, Mc Master, G, Coll, JL, Heitz, F, and Divita, G (2009). Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth. *Nucleic Acids Res* **37**: 4559–4569.
170. Hatakeyama, H, Ito, E, Akita, H, Oishi, M, Nagasaki, Y, Futaki, S, and Harashima, H (2009). A pH-sensitive fusogenic peptide facilitates endosomal escape and greatly enhances the gene silencing of siRNA-containing nanoparticles in vitro and in vivo. *Journal of controlled release* **139**: 127–132.
171. Kim, WJ, Christensen, LV, Jo, S, Yockman, JW, Jeong, JH, Kim, YH, and Kim, SW (2006). Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. *Mol Ther* **14**: 343–350.
172. Kumar, P, Wu, H, McBride, JL, Jung, KE, Kim, MH, Davidson, BL, Lee, SK, Shankar, P, and Manjunath, N (2007). Transvascular delivery of small interfering RNA to the central nervous system. *Nature* **448**: 39–43.
173. Eguchi, A, Meade, BR, Chang, YC, Fredrickson, CT, Willert, K, Puri, N, and Dowdy, SF (2009). Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. *Nat Biotechnol* **27**: 567–571.
174. Michiue, H, Eguchi, A, Scadeng, M, and Dowdy, SF (2009). Induction of in vivo synthetic lethal RNAi responses to treat glioblastoma. *Cancer Biol Ther* **8**.
175. EL Andaloussi, S, Lehto, T, Mäger, I, Rosenthal-Aizman, K, Oprea, II, Simonson, OE, Sork, H, Ezzat, K, Copolovici, DM, Kurrikoff, K, Viola, JR, Zaghloul, EM, Sillard, R, Johansson, HJ, Said Hassane, F, Guterstam, P, Suhorutsenko, J, Moreno, PM, Oskolkov, N, Hälldin, J, Tedebark, U, Metspalu, A, Lebleu, B, Lehtio, J, Smith, CIE, and Langel, Ü (2011). Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. *Nucleic Acids Res*. In press.
176. Turner, JJ, Jones, S, Fabani, MM, Ivanova, G, Arzumanov, AA, and Gait, MJ (2007). RNA targeting with peptide conjugates of oligonucleotides, siRNA and PNA. *Blood Cells Mol Dis* **38**: 1–7.
177. Moschos, SA, Jones, SW, Perry, MM, Williams, AE, Erjefalt, JS, Turner, JJ, Barnes, PJ, Sproat, BS, Gait, MJ, and Lindsay, MA (2007). Lung delivery studies using siRNA conjugated to TAT(48–60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. *Bioconjug Chem* **18**: 1450–1459.
178. Muratovska, A, and Eccles, MR (2004). Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. *FEBS Lett* **558**: 63–68.

18

179. Davidson, TJ, Harel, S, Arboleda, VA, Prunell, GF, Shelanski, ML, Greene, LA, and Troy, CM (2004). Highly efficient small interfering RNA delivery to primary mammalian neurons induces MicroRNA-like effects before mRNA degradation. *J Neurosci* **24**: 10040–10046.
180. Ramsay, E, and Gumbleton, M (2002). Polylysine and polyornithine gene transfer complexes: A study of complex stability and cellular uptake as a basis for their differential in-vitro transfection efficiency. *J Drug Target* **10**: 1–9.
181. Rothbard, JB, Kreider, E, VanDeusen, CL, Wright, L, Wylie, BL, and Wender, PA (2002). Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake. *J Med Chem* **45**: 3612–3618.
182. Wender, PA, Mitchell, DJ, Pattabiraman, K, Pelkey, ET, Steinman, L, and Rothbard, JB (2000). The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc Natl Acad Sci U S A* **97**: 13003–13008.
183. Merrifield, RB (1963). Solid Phase Peptide Synthesis. 1. The synthesis of a tetrapeptide. *J Am Chem Soc* **85**: 2149–2154.
184. Crombez, L, Morris, MC, Deshayes, S, Heitz, F, and Divita, G (2008). Peptide-Based Nanoparticle for Ex Vivo and In Vivo Dug Delivery. *Curr Pharm Design* **14**: 3656–3665.
185. Harris, TJ, Green, JJ, Fung, PW, Langer, R, Anderson, DG, and Bhatia, SN (2010). Tissue-specific gene delivery via nanoparticle coating. *Biomaterials* **31**: 998–1006.
186. Viola, JR, Leijonmarck, H, Simonson, OE, Oprea, II, Frithiof, R, Purhonen, P, Moreno, PM, Lundin, KE, Stromberg, R, and Smith, CIE (2009). Fatty acid-spermine conjugates as DNA carriers for nonviral in vivo gene delivery. *Gene Ther* **16**: 1429–1440.
187. Kang, SH, Cho, MJ, and Kole, R (1998). Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. *Biochemistry* **37**: 6235–6239.
188. Collingwood, MA, Rose, SD, Huang, L, Hillier, C, Amarzguioui, M, Wiiger, MT, Soifer, HS, Rossi, JJ, and Behlke, MA (2008). Chemical modification patterns compatible with high potency dicer-substrate small interfering RNAs. *Oligonucleotides* **18**: 187–200.
189. Nguyen, LT, Atobe, K, Barichello, JM, Ishida, T, and Kiwada, H (2007). Complex formation with plasmid DNA increases the cytotoxicity of cationic liposomes. *Biol Pharm Bull* **30**: 751–757.
190. Wolff, JA, and Budker, V (2005). The mechanism of naked DNA uptake and expression. *Adv Genet* **54**: 3–20.
191. Bello-Roufai, M, Lambert, O, and Pitard, B (2007). Relationships between the physicochemical properties of an amphiphilic triblock copolymers/DNA complexes and their intramuscular transfection efficiency. *Nucleic Acids Res* **35**: 728–739.
192. Pitard, B, Pollard, H, Agbulut, O, Lambert, O, Vilquin, JT, Cherel, Y, Abadie, J, Samuel, JL, Rigaud, JL, Menoret, S, Anegon, I, and Escande, D (2002). A nonionic amphiphile agent promotes gene delivery in vivo to skeletal and cardiac muscles. *Hum Gene Ther* **13**: 1767–1775.
193. Namgung, R, Nam, S, Kim, SK, Son, S, Singha, K, Kwon, JS, Ahn, Y, Jeong, MH, Park, IK, Garripelli, VK, Jo, S, and Kim, WJ (2009). An acid-labile

temperature-responsive sol-gel reversible polymer for enhanced gene delivery to the myocardium and skeletal muscle cells. *Biomaterials* **30**: 5225–5233.
194. Mäe, M, EL Andaloussi, S, Lehto, T, and Langel, Ü (2009). Chemically modified cell-penetrating peptides for the delivery of nucleic acids. *Expert Opin Drug Deliv* **6**: 1195–1205.
195. McCaffrey, AP, Meuse, L, Pham, TT, Conklin, DS, Hannon, GJ, and Kay, MA (2002). RNA interference in adult mice. *Nature* **418**: 38–39.
196. Tao, W, Davide, JP, Cai, M, Zhang, GJ, South, VJ, Matter, A, Ng, B, Zhang, Y, and Sepp-Lorenzino, L (2010). Noninvasive imaging of lipid nanoparticle-mediated systemic delivery of small-interfering RNA to the liver. *Mol Ther* **18**: 1657–1666.
197. Akinc, A, Goldberg, M, Qin, J, Dorkin, JR, Gamba-Vitalo, C, Maier, M, Jayaprakash, KN, Jayaraman, M, Rajeev, KG, Manoharan, M, Koteliansky, V, Rohl, I, Leshchiner, ES, Langer, R, and Anderson, DG (2009). Development of lipidoid-siRNA formulations for systemic delivery to the liver. *Mol Ther* **17**: 872–879.
198. Love, KT, Mahon, KP, Levins, CG, Whitehead, KA, Querbes, W, Dorkin, JR, Qin, J, Cantley, W, Qin, LL, Racie, T, Frank-Kamenetsky, M, Yip, KN, Alvarez, R, Sah, DW, de Fougerolles, A, Fitzgerald, K, Koteliansky, V, Akinc, A, Langer, R, and Anderson, DG (2010). Lipid-like materials for low-dose, in vivo gene silencing. *Proc Natl Acad Sci U S A* **107**: 1864–1869.
199. Semple, SC, Akinc, A, Chen, J, Sandhu, AP, Mui, BL, Cho, CK, Sah, DW, Stebbing, D, Crosley, EJ, Yaworski, E, Hafez, IM, Dorkin, JR, Qin, J, Lam, K, Rajeev, KG, Wong, KF, Jeffs, LB, Nechev, L, Eisenhardt, ML, Jayaraman, M, Kazem, M, Maier, MA, Srinivasulu, M, Weinstein, MJ, Chen, Q, Alvarez, R, Barros, SA, De, S, Klimuk, SK, Borland, T, Kosovrasti, V, Cantley, WL, Tam, YK, Manoharan, M, Ciufolini, MA, Tracy, MA, de Fougerolles, A, MacLachlan, I, Cullis, PR, Madden, TD, and Hope, MJ (2010). Rational design of cationic lipids for siRNA delivery. *Nat Biotechnol* **28**: 172–176.

ACKNOWLEDGEMENTS

This work has been mostly carried out at the Institute of Technology at the University of Tartu and at the Department of Neurochemistry at the Stockholm University. This work was supported by Swedish Research Council (VR-NT); by Center for Biomembrane Research, Stockholm; by Knut and Alice Wallenberg's Foundation; by the EU through the European Regional Development Fund through the Center of Excellence in Chemical Biology, Estonia; by the targeted financing SF0180027s08 from the Estonian Government; by the DoRa Program of The European Social Fund; and by Archimedes Foundation

First of all I would like to thank my supervisor Prof. Ülo Langel for giving me an opportunity to do my PhD in his group. It has been a great pleasure to work under your supervision and I am very glad that I have been introduced to the field of cell-penetrating peptides.

Secondly, I want to thank two of my close co-workers, Samir EL Andaloussi and Imre Mäger. Samir, you have been such an inspiration in science and have played big part in my development. Imre, your skills have been so invaluable and I appreciate the constant opportunity to discuss every little detail in science. Last but not least I thank you both for being such a good friends.

I would like to thank our group in Tartu: Kent Langel, Indrek Saar, Helena Sork, Kaido Kurrikoff, Nikita Oskolkov, Elo Eriste, Dana Copolovici, Julia Suhorutšenko, Piret Arukuusk and the students. It has been a pleasure to work with you.

I want to thank present and former co-workers at the Department of Neurochemistry at the Stockholm University: Kariem Ezzat, Peter Guterstam, Andrés Muñoz Alarcón, Karolina Wahlström, Maarja Mäe, Anderś Floren, Marie-Louise Tjörnhammer, Rannar Sillard, Katri Rosenthal-Aizman, Mattias Hällbrink, Henrik Johansson and Yang Jiang.

I want to thank our collaborators. At the Department of Laboratory Medicine at the Karolinska Institutet: Prof. Edvard Smith, Oscar Simonson, Joana Viola, Eman Zaghloul, Per Lundin and Pedro Moreno. At the Institute of Technology at the University of Tartu: Prof. Andres Merits, Liane Ülper and Gregory Heller. At the University of Montpellier: Prof. Bernard Lebleu, Rachida Abes and Fatouma Said Hassane. At the Institute of Molecular and Cell Biology at the University of Tartu: Prof. Margus Pooga, Pille Säälik and Kärt Padari.

Finally, I would like to thank people who proof-read my thesis: Samir, Pille, Kaido and Imre.

Suur tänu mulle lähedastele inimestele.

PUBLICATIONS

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List of publications

1. Mäe, M, EL Andaloussi, S, **Lehto, T,** and Langel, Ü (2009). Chemically modified cell-penetrating peptides for the delivery of nucleic acids. *Exp Opin Drug Delivery*. **6**: 1195–1205.
2. **Lehto, T,** Abes, R, Oskolkov, N, Suhorutšenko, J, Copolovici, DM, Mäger, I, Viola, JR, Simonson, OE, Ezzat, K, Guterstam, P, Eriste, E, Smith, CIE, Lebleu, B, EL Andaloussi, S, and Langel, Ü (2010). Delivery of nucleic acids with a stearylated $(RxR)_4$ peptide using a non-covalent co-incubation strategy. *J Control Release.* **141**: 42–51.
3. Eiríksdóttir, E, Mäger, I, **Lehto, T,** El Andaloussi, S, and Langel, Ü (2010). Cellular internalization kinetics of (luciferin-)cell-penetrating peptide conjugates. *Bioconjug Chem.* **21**: 1662–72.
4. El Andaloussi, S, **Lehto, T,** Lundin, P, and Langel, Ü (2010). Application of PepFect peptides for the delivery of splice correcting oligonucleotides. *Methods Mol Biol.* **683**: 361–73.

5. **Lehto, T,** Simonson, OE, Mäger, I, Ezzat, K, Sork, H, Copolovici, DM, Viola, JR, Zaghloul, EM, Lundin, P, Moreno, PMD, Mäe, M, Oskolkov, N, Suhorutšenko, J, Smith, CIE, and EL Andaloussi, S (2011). A peptide-based vector for efficient gene transfer *in vitro* and *in vivo*. *Mol Ther.* In press.
6. EL Andaloussi, S, **Lehto, T,** Mäger, I, Rosenthal-Aizman, K, Oprea, II, Simonson, OE, Sork, H, Ezzat, K, Copolovici, DM, Kurrikoff, K, Viola, JR, Zaghloul, EM, Sillard, R, Johansson, HJ, Hassane, FS, Guterstam, P, Suhorutšenko, J, Moreno, PMD, Oskolkov, N, Hälldin, J, Tedebark, U, Metspalu, A, Lebleu, B, Lehtiö, J, Smith, CIE, and Langel, Ü (2011). Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically *in vivo*. *Nucleic Acids Res*. In press.
7. Ezzat, K, EL Andaloussi, S, Zaghloul, EM, **Lehto, T,** Lindberg, S, Magdy, T, Viola, JR, Guterstam, P, Moreno, PMD, Abdo, R, Sillard, R, Hammond, SM, Wood, MJA, Arzumanov, AA, Gait, MJ, Smith, CIE, Hällbrink, M, and Langel, Ü (2011). PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. *Nucleic Acids Res*. In press.
8. Hassane, FS, Abes, R, EL Andaloussi, S, **Lehto, T,** Sillard, R, Langel, Ü, and Lebleu, B (2011). Insights into the intracellular trafficking of Transportan and RXR cell penetrating peptides derivatives. *J Control Release.* In press.

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Teaduspublikatsioonid

1. Mäe, M, EL Andaloussi, S, **Lehto, T,** and Langel, Ü (2009). Chemically modified cell-penetrating peptides for the delivery of nucleic acids. *Exp Opin Drug Delivery*. **6**: 1195–1205.
2. **Lehto, T,** Abes, R, Oskolkov, N, Suhorutšenko, J, Copolovici, DM, Mäger, I, Viola, JR, Simonson, OE, Ezzat, K, Guterstam, P, Eriste, E, Smith, CIE, Lebleu, B, EL Andaloussi, S, and Langel, Ü (2010). Delivery of nucleic acids with a stearylated $(RxR)_4$ peptide using a non-covalent co-incubation strategy. *J Control Release.* **141**: 42–51.
3. Eiríksdóttir, E, Mäger, I, **Lehto, T,** El Andaloussi, S, and Langel, Ü (2010). Cellular internalization kinetics of (luciferin-)cell-penetrating peptide conjugates. *Bioconjug Chem.* **21**: 1662–72.
4. El Andaloussi, S, **Lehto, T,** Lundin, P, and Langel, Ü (2010). Application of PepFect peptides for the delivery of splice correcting oligonucleotides. *Methods Mol Biol.* **683**: 361–73.
5. **Lehto, T,** Simonson, OE, Mäger, I, Ezzat, K, Sork, H, Copolovici, DM, Viola, JR, Zaghloul, EM, Lundin, P, Moreno, PMD, Mäe, M, Oskolkov, N,

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Suhorutšenko, J, Smith, CIE, and EL Andaloussi, S (2011). A peptide-based vector for efficient gene transfer *in vitro* and *in vivo*. *Mol Ther.* In press.

6. EL Andaloussi, S, **Lehto, T,** Mäger, I, Rosenthal-Aizman, K, Oprea, II, Simonson, OE, Sork, H, Ezzat, K, Copolovici, DM, Kurrikoff, K, Viola, JR, Zaghloul, EM, Sillard, R, Johansson, HJ, Hassane, FS, Guterstam, P, Suhorutšenko, J, Moreno, PMD, Oskolkov, N, Hälldin, J, Tedebark, U, Metspalu, A, Lebleu, B, Lehtiö, J, Smith, CIE, and Langel, Ü (2011). Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically *in vivo*. *Nucleic Acids Res*. In press.
7. Ezzat, K, EL Andaloussi, S, Zaghloul, EM, **Lehto, T,** Lindberg, S, Magdy, T, Viola, JR, Guterstam, P, Moreno, PMD, Abdo, R, Sillard, R, Hammond, SM, Wood, MJA, Arzumanov, AA, Gait, MJ, Smith, CIE, Hällbrink, M, and Langel, Ü (2011). PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. *Nucleic Acids Res*. In press.
8. Hassane, FS, Abes, R, EL Andaloussi, S, **Lehto, T,** Sillard, R, Langel, Ü, and Lebleu, B (2011). Insights into the intracellular trafficking of Transportan and RXR cell penetrating peptides derivatives. *J Control Release.* In press.

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

1. **Imre Mäger.** Characterization of cell-penetrating peptides: Assessment of cellular internalization kinetics, mechanisms and bioactivity. Tartu 2011, 132 p.