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Design and application of RNA therapeutics for splice site mutations

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Design and application of RNA therapeutics for splice site mutations
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution. It is, strictly speaking, a real factor in scientific research."

— Albert Einstein, On Cosmic Religion and Other Opinions and Aphorisms

To my mother and father…

Anneme ve babama…

ABSTRACT

Precursor messenger RNA splicing is one of the most fundamental and complex mechanisms in eukaryotes. Over 90% of the human genes undergo alternative splicing, which is essential for the regulation of gene expression. A dynamic RNA-protein complex called the spliceosome catalyzes splicing. The spliceosome recognizes core splice site signals, and its function is furthermore regulated by other sequence elements that can either silence or enhance splicing in a cell-specific manner. These regulatory elements are recognized by trans-acting protein factors which modulate the function of the spliceosome. However, the fact that splicing is one of the most regulated mechanisms in the cell also makes it prone to dysfunctions caused by mutations.

Mis-splicing diseases account for up to 30% of the inherited genetic diseases. They can be caused by mutations in the core splice sites as well as in the regulatory elements. Mutations that disrupt the splicing mechanism may result in RNA degradation, non-functional protein products, or toxic proteins that might alter the cellular environment. The understanding of mis-splicing disease mechanisms has been subject to various studies aiming at finding the appropriate therapeutics.

In parallel with the development of gene therapy, where the classical aim is to introduce the corrected gene to cure a disease, another field has emerged; antisense oligonucleotide therapeutics. These RNA-DNA-based oligonucleotides can be designed to alter gene expression as well as to manipulate the splicing mechanism. Since their emergence during 1970s, antisense oligonucleotides have been extensively studied within the mis-splicing disease field, with several clinical trials ongoing.

In this thesis, in paper I, we report a novel solid-phase synthesis method with a biological proof-of-concept. This synthesis method allows the conjugation of therapeutic oligonucleotides via a cleavable disulfide linker. The developed method can be applied to target several transcripts or different parts within the same transcript by allowing delivery of equimolar amounts of therapeutics. In paper II, we explore the possibility of using splice-correction approach for restoring the aberrant splicing of the gene *BTK*. Lack of *BTK* causes a primary immunodeficiency disease called XLA, which is a B cell developmental disorder. For the first time, we show splice-correction in B cells by modified oligonucleotide therapeutics both ex vivo and in vivo. In paper III, we aim at developing methods to rescue the core splice site mutations in *BTK* by using bifunctional oligonucleotide therapeutics. These oligonucleotides have the ability to recruit splice factor proteins, improving the splicing of mutated sites. We show that rescuing of core splice site mutations is possible yet it has its own challenges, which need to be taken into account in the design process. The results in this thesis have provided new therapeutics for a genetic disease, and more generally explores new methods for improving the function and delivery of oligonucleotide therapeutics.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles:

I. **Bestas B*,** McClorey G*, Tedebark U, Moreno PM, Roberts TC, Hammond SM, Smith CI, Wood MJ, and Andaloussi SE. Design and application of bispecific splice-switching oligonucleotides.
Nucleic Acid Ther. 2014;24(1):13-24.

II. **Bestas B***, Moreno PM*, Blomberg KE, Mohammad DK, Saleh AF, Sutlu T, Nordin JZ, Guterstam P, Gustafsson MO, Kharazi S, Piatosa B, Roberts TC, Behlke MA, Wood MJ, Gait MJ, Lundin KE, El Andaloussi S, Månsson R, Berglöf A, Wengel J, and Smith CIE. Splice-correcting oligonucleotides restore BTK function in X-linked agammaglobulinemia model.
The Journal of Clinical Investigation. 2014;124(9):4067-81.

III. Turunen JJ*, **Bestas B***, Tedebark U, Wengel J and Smith CIE. Parameters for correcting exon-skipping splicing defects in the BTK mRNA by the use of bifunctional oligonucleotides. (Manuscript)

* *Equal Contribution*

Other publications by the author not included in this thesis:

I. Zaghloul EM, Madsen AS, Moreno PM, Oprea, II, El-Andaloussi S, **Bestas B**, Gupta P, Pedersen EB, Lundin KE, Wengel J, and Smith CIE. Optimizing anti-gene oligonucleotide 'Zorro-LNA' for improved strand invasion into duplex DNA.
Nucleic Acids Res. 2011;39(3):1142-54.

II. Jezowska M, Romanowska J, **Bestas B**, Tedebark U, and Honcharenko M. Synthesis of biotin linkers with the activated triple bond donor [p-(N-propynoylamino)toluic acid] (PATA) for efficient biotinylation of peptides and oligonucleotides.
Molecules. 2012;17(12):14174-85.

III. Moreno PM, Geny S, Pabon YV, Bergquist H, Zaghloul EM, Rocha CS, Oprea, II, **Bestas B**, Andaloussi SE, Jorgensen PT, Pedersen EB, Lundin KE, Zain R, Wengel J, and Smith CIE. Development of bis-locked nucleic acid (bisLNA) oligonucleotides for efficient invasion of supercoiled duplex DNA.
Nucleic Acids Res. 2013;41(5):3257-73.

IV. Honcharenko M, Zytek M, **Bestas B**, Moreno P, Jemielity J, Darzynkiewicz E, Smith CIE, and Strömberg R. Synthesis and evaluation of stability of m3G-CAP analogues in serum-supplemented medium and cytosolic extract.
Bioorganic & Medicinal Chemistry. 2013;21(24):7921-8.

V. Berglöf A, Turunen JJ, Gissberg O, **Bestas B**, Blomberg KE, and Smith CIE. Agammaglobulinemia: causative mutations and their implications for novel therapies.
Expert Rev Clin Immunol. 2013;9(12):1205-21.

VI. **Bestas B**, Turunen JJ, Blomberg KE, Wang Q, Månsson R, El Andaloussi S, Berglöf A, and Smith CIE. Splice-correction strategies for treatment of x-linked agammaglobulinemia.
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LIST OF ABBREVIATIONS

2'-OMe	2'-*O*-methyl
3' ss	3' splice site
5' ss	5' splice site
Ago2	Argonaute 2
AMO	anti-miRNA AON
AON	antisense oligonucleotide
APOB	apolipoprotein B
BAC	Bacterial Artificial Chromosome
BCR	B cell receptor
BMD	Becker muscular dystrophy
BPS	branch point sequence
BTK	Bruton’s Tyrosine Kinase
CPP	cell-penetrating peptide
CRISPR	clustered regularly interspaced short palindromic repeat
DM	myotonic dystrophy
DMD	Duchenne muscular dystrophy
DMPK	dystrophia myotonica-protein kinase
dsRNA	double-stranded RNA
EJC	exon junction complex
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
hnRNP	heterogeneous nuclear ribonucleoprotein
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
KO	knock-out
LNA	Locked Nucleic Acid
MAPT	microtubule associated protein tau
miRNA	microRNA
MLV	Moloney murine leukemia virus
MOE	2'-*O*-methoxyethyl
mRNA	messenger RNA
Mstn	Myostatin

NMD	nonsense-mediated decay
NPC1	Niemann–Pick disease type C1
nt	nucleotide(s)
PH	Pleckstrin homology
Pip	PMO/PNA internalizing peptide
PLCγ2	phospholipase Cγ2
PMO	phosphorodiamidate morpholino oligomer
PNA	peptide nucleic acid
PPT	polypyrimidine tract
pre-mRNA	precursor mRNA
pri-miRNAs	primary micro-RNAs
PS	phosphorothioate
PTC	premature termination codon
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNP	ribonucleoprotein
SCID	severe combined immunodeficiency
SH3	Src homology 3
siRNA	small interfering RNA
SMA	spinal muscular atrophy
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SR	serine-arginine (rich)
TALEs	transcription activator-like effectors
TDP-43	TAR DNA-binding protein 43
TH	Tec homology
TIA-1	T-cell intracellular antigen 1
TRPBP	TAR-RNA binding protein
U2AF	U2 auxiliary factor
UNA	Unlocked Nucleic Acid
xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia
ZFP	zinc-finger proteins

1 INTRODUCTION

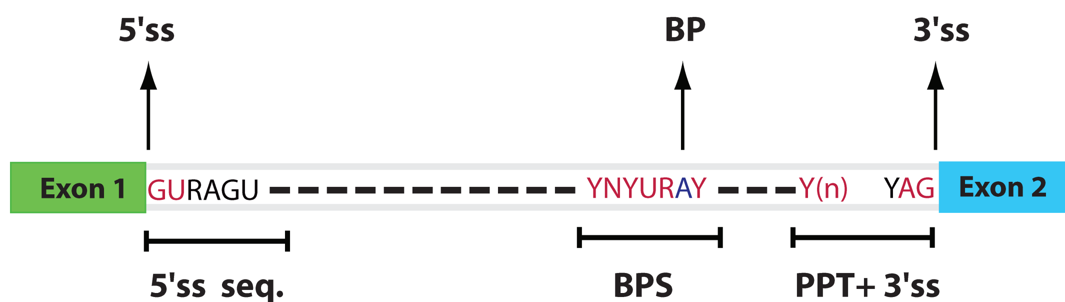
1.1 Pre-mRNA splicing and the spliceosome

Since the discovery of the double helix structure of DNA in 1953 (Brenner et al., 1961; Watson and Crick, 1953), the understanding of the RNA world has evolved immensely. Now we know much more about the regulatory function of RNA rather than thinking of it as just an intermediate product for protein production (Morris and Mattick, 2014). During the 1970s, it was discovered that genes are not continuous but rather split by intronic regions and these regions do not exist in the mature messenger RNA (mRNA) (Berget et al., 1977; Chow et al., 1977; Gilbert, 1978). So how does the cell produce mRNA? Soon after, it was reported that isolated small nuclear RNAs (snRNAs) have base-pairing interactions with splice sites and this initiated the understanding that precursor RNA (pre-mRNA) splicing is processed by a ribonucleoprotein (RNP) complex (Lerner and Steitz, 1979), the spliceosome (Black and Pinto, 1989; Lerner et al., 1980; Mount et al., 1983; Rogers and Wall, 1980; Sharp, 2005).

The spliceosome is composed of a dynamic RNA/protein complex, which regulates the formation of the mature mRNA. Uridine-rich small nuclear RNAs (snRNAs) U1, U2, U5 U4/U6 are the main building blocks of the spliceosome and they complex with proteins to become small nuclear ribonucleoproteins (snRNPs). Apart from the specific snRNA-associated proteins, each of them has a heptameric protein ring complex composed of Sm proteins (LSm in the case of U6) (Jurica and Moore, 2003; Matera and Wang, 2014; Ritchie et al., 2009). In most metazoans and plants, and some unicellular organisms, there is also a distinct type of splicing machinery that recognizes a rare class of introns (U12-type) called the minor spliceosome. U12-type introns are processed by snRNPs U11, U12, U4atac, U6atac, and U5, which is common in both systems (Turunen et al., 2013).

The core splice sites are the 5' splice site (5'ss), 3' splice site (3'ss), polypyrimidine tract (PPT) and the branch point sequence (BPS) which is usually 18-40 nt upstream of the 3'ss (Wahl et al., 2009) **(Figure 1A)**. The assembly of the spliceosome begins with the binding of U1 snRNP to the 5'ss and this binding is usually stabilized by the serine-arginine rich (SR) protein factors in eukaryotes. Following this, SF1 binds to BPS and the heterodimer protein U2 auxiliary factor (U2AF) recognizes the PPT and the 3'ss via its protein domain. SF1 is later on replaced by U2 snRNP, and the tri-snRNP complex U4/U6.U5 is recruited to the 5'ss **(Figure 1B)**. In order to become catalytically active for the transesterification reactions, conformational and compositional remodeling occurs within the spliceosome that allows the adenosine in the BPS to come closer to the 5'ss for the first nucleophilic attack and eventually cutting away the intron (Wahl et al., 2009). Moreover, recent evidence proves that U6 snRNA forms the core of the active site during splicing (Fica et al., 2014; Fica et al., 2013).

A



B

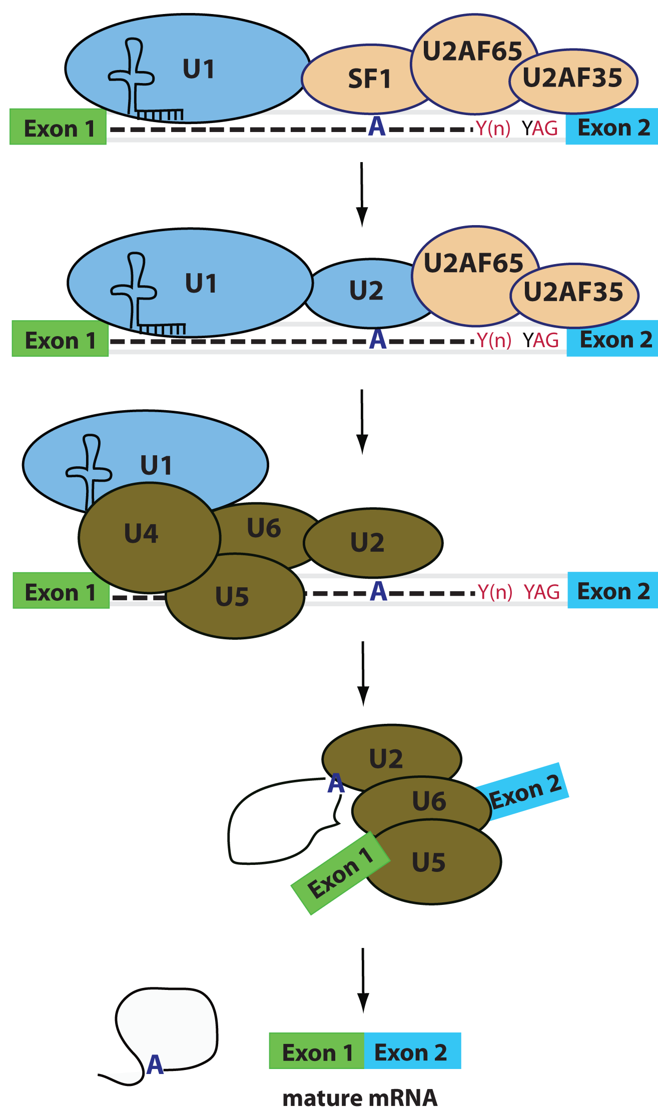


Figure 1. Core splicing signals and spliceosome assembly. A. Core splice site sequences. 5' splice site (5'ss), 3' splice site (3'ss), branch point sequence (BPS) and polypyrimidine tract (PPT). "N" is any nucleotide and "R" is for purines and "Y" is for pyrimidines. Branch point (BP) adenosine is depicted in blue. **B.** A simple scheme for spliceosome arrangement. Dashed line is for the intronic sequence.

Mass spectrometry analysis of different stages of the spliceosome assembly reveals that it is a highly dynamic process in which the inventory is changing in each step. The human spliceosome contains 45 distinct snRNP-related proteins and approximately 170 snRNP-unrelated proteins that are regulating splicing. This huge protein repertoire includes helicases, isomerases, kinases, phosphatases and ATPases that are helping to remodel the spliceosome. Moreover, posttranscriptional modifications such as acetylation, ubiqutinylation and phosphorylation of spliceosomal proteins also control RNA-RNA or RNA-protein binding during splicing (Wahl et al., 2009; Will and Luhrmann, 2011). During the recent years, there have been numerous studies to understand the higher order complexes of the spliceosome and its catalytic domains are being revealed by high-resolution structural techniques (Galej et al., 2014; Zhang et al., 2013).

In vertebrates, the average size of exons is 145bp with a median value of 122bp whereas average size of introns is 3365bp with a median value of 1023bp (Lander et al., 2001). When the intron size exceeds 200-250 nucleotides (nt), the step-wise assembly of the spliceosome directly on the intron reduces (Fox-Walsh et al., 2005). In this case, it is easier for the spliceosome first to recognize the exons, and this phenomenon is called "exon definition" (Berget, 1995). According to this model, exon recognition complex is assembled with U1 snRNP binding to the 5'ss and it promotes the binding of U2AF to the upstream 3'ss and the PPT which also recruits U2 snRNP to the BPS site. Meanwhile, serine-arginine rich (SR) proteins stabilize the complex **(Figure 2)**. The cross-connection between the exon definition complex and the intron definition components is not very well understood yet it may involve the cis-acting elements eventually connecting these two events (De Conti et al., 2013).

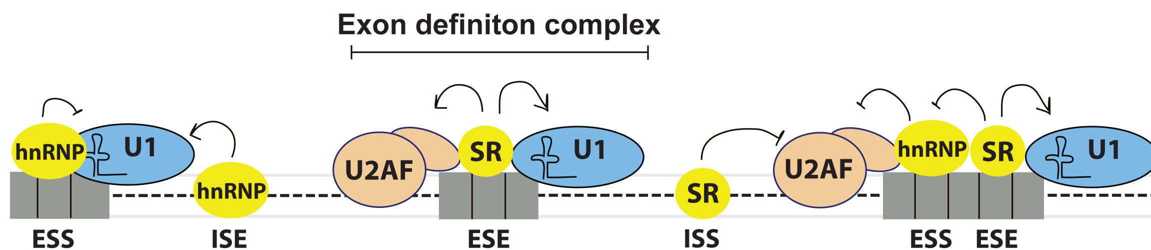


Figure 2. Exon definition complex and the splicing regulatory elements. Interactions between the spliceosome components and trans-acting regulatory factors are depicted; arrows represent activating interaction and blocked arrows represent the silencing interaction. Cis-regulatory sequences are marked within exons (ESE and ESS) and within introns (ISE and ISS). Dashed line is for the intronic sequence.

The core splice sites in the human genome are highly degenerate and there are numerous pseudo splice sites that surround exon-like sequences in the intronic regions. So how does the spliceosome decide which splice site sequences to bind to? One of the parameters that regulate splicing are cis-regulatory elements of the pre-mRNA, namely exonic splicing enhancers (ESE) or exonic splicing silencers (ESS) and intronic splicing enhancers (ISE) or intronic splicing silencers (ISS) (Wang and Burge, 2008) **(Figure 2)**. These are short sequences (around 6nt) on pre-mRNA, and trans-acting regulatory splice factor proteins recognize them in order to control the recognition of core splice sites by the spliceosome. One group of well-known trans-acting proteins is the SR proteins that usually act through the

ESEs and preferentially bind to purine rich sequences. SR proteins also recruit U1 and U2 snRNPs and the U2AF via their serine-arginine repeat motifs during the early assembly of the spliceosome. Other trans-acting splicing factors such as the heterogeneous nuclear ribonucleoprotein (hnRNP) family proteins can act on silencer sequences either by creating steric hindrance for the spliceosome or by activating conformational changes on the pre-mRNA, yet in some cases they can also act as enhancers (De Conti et al., 2013; Lee and Rio, 2015; Singh and Valcarcel, 2005). There is a huge context dependency of these factors throughout splicing so there are no clear rules about their enhancing or silencing activities. An example of this phenomenon is hnRNP H, which binds to intronic G-rich sequences to promote splicing however when bound to exonic regions, it represses splicing (Fu and Ares, 2014) **(Figure 2)**. Other examples of similar trans-acting factors are TAR DNA-binding protein 43 (TDP-43) and T-cell intracellular antigen 1 (TIA-1), also exploited in this thesis. TIA-1 has been reported to interact with the intronic U-rich sequences downstream of the 5'ss and recruit U1 snRNP for splice site recognition (Forch et al., 2002; Wang et al., 2010). TDP-43 has been reported to enhance splicing of an exon with a weak 5'ss through binding to the (UG) repeats in proximity, though the authors also note that this is not a universal rule and depends on the precise context (Passoni et al., 2012).

To this end, another important parameter in the regulation of splicing is the pre-mRNA structure (McManus and Graveley, 2011). Secondary structures in pre-mRNA might sequester core splice sites, block the binding of snRNAs or bring splice sites in close proximity (Warf and Berglund, 2010). It was found that secondary structures are more conserved around alternatively spliced exons (Shepard and Hertel, 2008). Moreover, it was also reported that splice enhancer and silencer regions are usually single-stranded which might be required for the trans-factors to recognize these regions (Hiller et al., 2007). So all these regulations affect the binding of snRNPs and trans-acting factors to the pre-mRNA in order to control the identification of correct splice sites, in a cell-type specific manner.

Two ways of splicing are typically considered separately; constitutive splicing and alternative splicing. Constitutive splicing is the process where all the exons are joined in order, whereas alternative splicing is the processing of the pre-mRNA by differential usage of the splice sites by the help of cis- and trans-acting elements **(Figure 3)**. Alternative splicing enables the synthesis of various transcripts from one gene and enhances the protein diversity of complex organisms, which is important for cell survival, differentiation and many other crucial pathways in the cell (Kelemen et al., 2013). Alternative splicing depends on transcription rate, tissue specific factors, post-transcriptional modification of splice-factors, chromatin structure, non-coding RNAs, intron size, and the selection of core splice sites (Chen and Manley, 2009; de Klerk and t Hoen, 2015; Nilsen and Graveley, 2010).

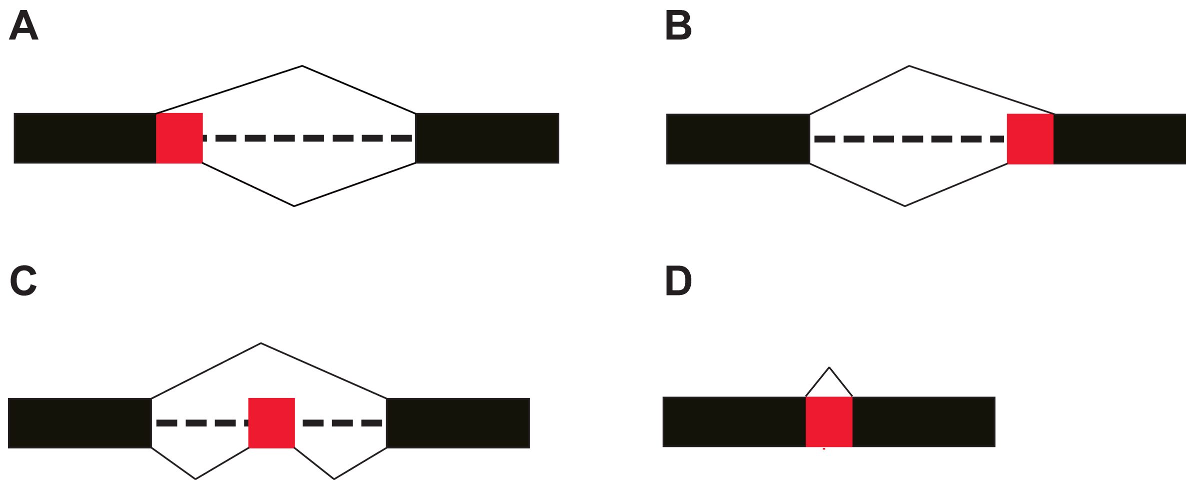


Figure 3. Types of alternative splicing. A. Alternative 5'ss splicing. **B.** Alternative 3'ss splicing. **C.** Alternative exon inclusion or exclusion. **D.** Intron retention. Red boxes represent the alternative exons either included or excluded. Dashed lines represent the intronic regions.

The "splicing code" is highly complex and integrated understanding of molecular and structural information is required to solve the code and create a biophysical model (Arias et al., 2015; Barash et al., 2010). A lot of effort has been done both in silico and in vitro in order to predict the behavior of RNA transcripts and so "to decipher the code". A recently published mathematical model provides predictions on exon inclusion within different tissues taking into consideration features such as splice site signals, secondary structure, exon and intron lengths, regulatory sequences, frequency of mono-, di-, tri- nucleotide sequences as well as context dependency. The tool can also take into account single nucleotide variants and predict exon inclusion. The authors created this model via the concept "machine learning" where they collected information from healthy human tissues. Although it needs more parameters to consider, these types of approaches will pave the way for designing therapies for splicing mutations (Guigo and Valcarcel, 2015; Xiong et al., 2015). In the next section, I will discuss the changes in the splicing code that result in deleterious effects.

1.2 Dysregulation of splicing

The challenge of the spliceosome is to recognize the correct exons within long introns and perform the splicing in the correct order according to the need of the cell. It is reported that over 90% of human protein-coding transcripts undergo alternative splicing (Pan et al., 2008). This highly variable mechanism and its regulation via different elements make it also prone to errors via mutations. These mutations might occur within the core splice sites, cis-regulatory elements or in trans-acting factors (Ward and Cooper, 2010). Human Genome Mutation Database (HGMD) reports that 10-15% of disease causing SNPs are within the splice sites (Stenson et al., 2014) and 20% of the exonic missense mutations affect the splicing elements (Sterne-Weiler and Sanford, 2014). Mutations might cause exon skipping, create *de novo* cis-regulatory elements, create cryptic splice sites, enhance the splicing of pseudoexons, affect RNA stability and create toxic RNA or protein (Cooper et al., 2009; Singh and Cooper, 2012; Tazi et al., 2009). Mutations that affect the exonic or intronic cis-regulatory elements mostly cause exon exclusion, which might result in a frame-shift with a premature termination codon (PTC), usually causing nonsense-mediated decay (NMD). In

addition, activation of cryptic splice sites and pseudoexon inclusion might also result in frameshifts and degradation. NMD is a quality control mechanism and clears away the RNA transcripts that have PTCs in order to avoid the build-up of “wrong” transcripts in the cell. The most well-known mechanism of NMD is the activation of mRNA degradation when there is a stop codon >50-55 nucleotides before an exon junction complex (EJCs), a protein complex that marks the boundaries of joined exons (Karam et al., 2013; Schweingruber et al., 2013).

A good example of the mutations affecting the cis-regulatory elements is the spinal muscular atrophy (SMA), which is a fatal, motor neurodegenerative disease that is caused by loss-of-function mutations in the *SMN1* gene. There are two *SMN* genes in humans (1 and 2) and SMA patients still retain *SMN2*. The difference between these two genes is the single nucleotide change (C to T) at position 6 in exon 7 of the *SMN2* gene, which alters the splicing of exon 7, leading to truncated non-functional protein in 90% of the transcripts **(Figure 4A)**. The underlying mechanism has been explained with two models. First model suggests that the mutation disrupts the ESE where SRSF1 binds inside the exon or it creates ESS so that suppressor hnRNP A1 binds and inhibits exon recognition (Tazi et al., 2009). SMA is a crucial example where a one-nucleotide difference changes the nature of a regulatory sequence. The inclusion of exon 7 has been subject to design of oligonucleotide therapeutics, which will be explained in the last section (Porensky and Burghes, 2013; Rigo et al., 2014).

Another example comes from mutations resulting in RNA gain of function. When microsatellite repeats expand, they cause diseases. Myotonic dystrophy (DM) is an example in which CUG repeats located in 3' UTR of the dystrophia myotonica-protein kinase (DMPK) expands. MBNL1, an RNA metabolism regulator protein, is tethered by the expanded region and depleted from the nucleus and from other locations that need this protein **(Figure 4B)**. The expanded repeats form a structure resembling the MBNL1 natural binding site and sequester the protein that forms aggregates in the cell (Cooper et al., 2009). DM has been the subject for oligonucleotide therapeutics with the aim of targeting CUG repeats and releasing MBNL1 (Rigo et al., 2014).

Mutations can also alter alternative splicing and disrupt the ratio of the transcripts in the cell. An example is the *MAPT* gene (microtubule associated protein tau) where exon inclusion affects the isoform ratio. Exon 10 of *MAPT* spans enhancer and silencer regions that are controlling two different isoforms. When mutations fall into these regions they promote exon 10 inclusion **(Figure 4C)**. Tau protein (4R isoform) forms aggregates in the neuronal and glial cells, causing a neuropathological disorder related to Alzheimer’s disease (Tazi et al., 2009). *MAPT* has been subject to oligonucleotide-based therapeutics, yet still in early stages (Veltrop and Aartsma-Rus, 2014).

Activation of cryptic splice sites due to a silent mutation in the exon 11 of *LMNA* gene causes Hutchinson-Gilford progeria syndrome, a rare accelerated-aging syndrome (Rigo et al., 2014). The resulting transcript produces a shorter mutant protein **(Figure 4D)**. In addition, an example for pseudoexon inclusion is the Niemann–Pick disease type C1 (NPC1), a metabolic

disorder, where a point mutation in intron 9 strengthens the 3'ss and leads to the incorporation of the 194bp pseudoexon. This leads to PTC and NMD **(Figure 4E)**. Both of these syndromes have been subject to oligonucleotide-based therapeutics, including animal studies (Rigo et al., 2014; Rodriguez-Pascau et al., 2009; Scaffidi and Misteli, 2005).

In addition to the splice site mutations, mutations in the core-spliceosome components have been reported to occur especially in cancer. These types of alterations might change the intracellular concentration, localization or the composition of the required spliceosomal components, frequently changing the RNA transcript proportion in the cell (Pedrotti and Cooper, 2014). Over-expression of SR proteins has been observed in several cancer types and they have been reported to up-regulate the isoforms that are responsible for cell mobility and enhanced proliferation (Singh and Cooper, 2012). An example is the up-regulation of anti-apoptotic Bcl-x (L) isoform in cancer tissues that has been also subject to oligonucleotide based therapeutics (Bauman et al., 2010).

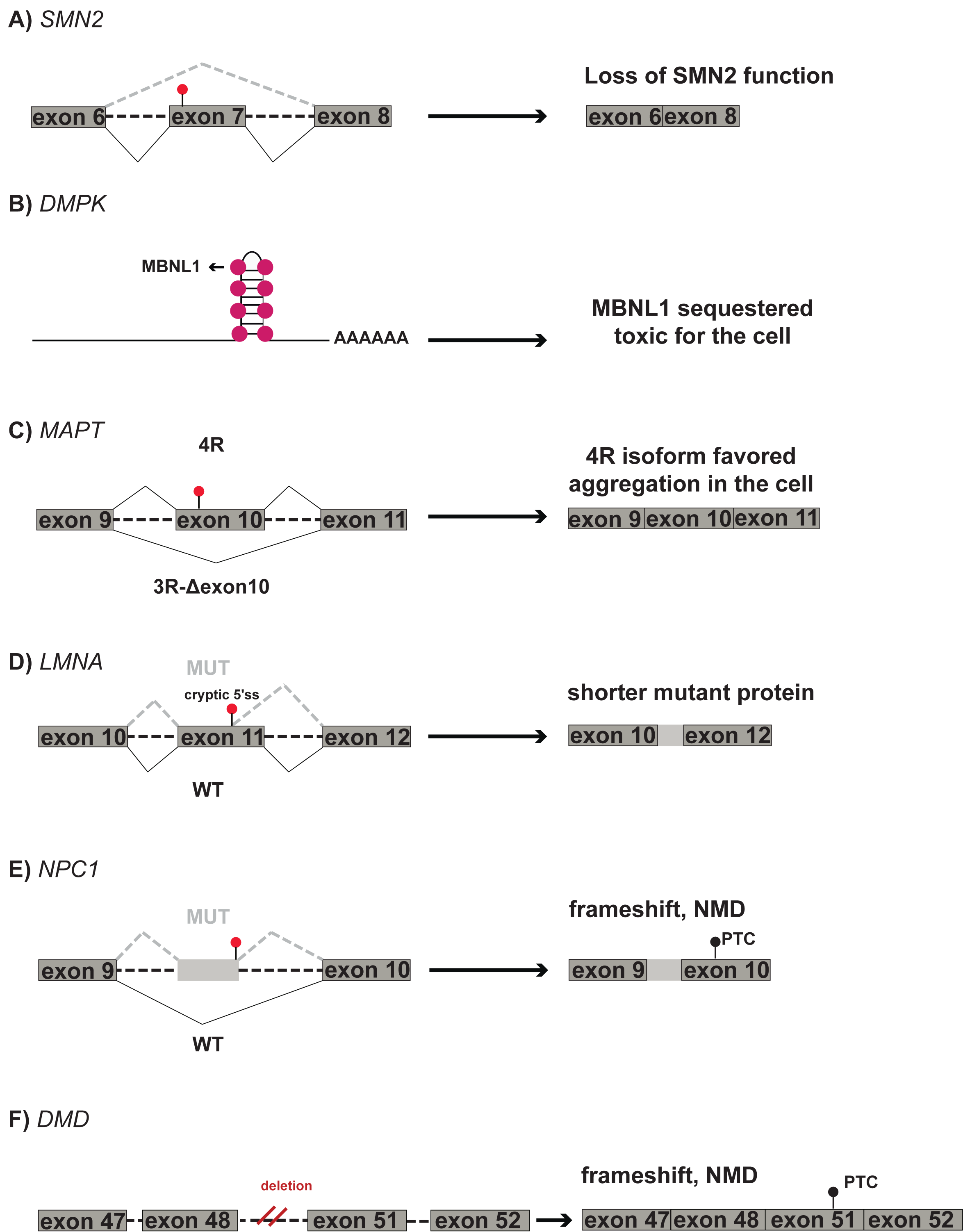


Figure 4. Mechanism of example diseases. A. Mutation in the *SMN2* gene causing exon 7 exclusion in spinal muscular atrophy (SMA). **B.** Expansion of CUG repeats in myotonic dystrophy (DM) results in the sequestering of protein MBNL1. **C.** Mutations in the *MAPT* gene change the isoform ratio in the cell. Uncontrolled synthesis of exon 10 included transcript (4R) causes aggregation of the protein tau. **D.** Mutation in exon 11 of *LMNA* gene activates a cryptic 5'ss resulting in the production of a shorter mutant protein, progerin. This causes Hutchinson-Gilford progeria syndrome. **E.** Mutation in the intron 9 of *NPC1* activates pseudoexon inclusion. This causes a frameshift with premature termination codon (PTC), resulting in nonsense-mediated decay (NMD). **F.** Deletions between the exons 48-51 of the *DMD* gene cause a frameshift and PTC in Duchenne muscular dystrophy (DMD). Black pin represents PTC and red pin represents mutation.

1.2.1 Example disease models for splicing therapy

In this section, I will briefly review two disease models, which are both studied in this thesis.

1.2.1.1 X-linked agammaglobulinemia (XLA)

Agammaglobulinemias are B cell deficiency diseases that are caused by the mutations in the genes responsible for the components of B cell receptor or its precursor (pre-B cell). X-linked agammaglobulinemia (XLA) accounts for 85% of this group (Berglöf et al., 2013). The disease was first described in 1952 (Bruton, 1952) and later on it was associated with the inactivation of the protein Bruton's Tyrosine Kinase (BTK) (Tsukada et al., 1993; Vetrie et al., 1993; Vihinen et al., 1999) which causes a developmental block from pro- to pre-B cell stage. Precursor B cells exist in the bone marrow (Nomura et al., 2000), however due to the inactivation of BTK, they cannot develop into further stages (Noordzij et al., 2002). As a result, patients have low percentage of peripheral B cells with an immature phenotype ($IgM^{high}IgD^{low}$) and they lack mature plasma cells that are responsible for antigen-specific Ig production (Conley, 1985; Sideras and Smith, 1995). Females are usually carriers of XLA with a healthy life, since due to the non-random X chromosome inactivation B cells that express the wild-type BTK have selective advantage (Conley and Puck, 1988).

XLA patients are vulnerable to bacterial and enteroviral infections and the most typical infections result from *Haemophilus influenza* and *Streptococcus pneumonia* (Plebani et al., 2002). Patients typically develop gastrointestinal and respiratory tract infections (Winkelstein et al., 2006). There is no curative treatment for XLA and the current treatment is the intravenous and subcutaneous substitution of immunoglobulins (IVIG) as a prophylaxis combined with the administration of antibiotics when infections occur. However, this is still not enough for the patients to be immune against all types of antigens, quality of life and life expectancy is reduced (Cunningham-Rundles, 2011; Gardulf et al., 1993; Winkelstein et al., 2006). Moreover IVIG treatment is very costly and not every patient can have access to such expensive treatment (Conley et al., 2009).

Mouse models have been the source for studying the mechanism of BTK in XLA. One of the widely used models is the X-linked immunodeficient (xid) mouse (Rawlings et al., 1993; Thomas et al., 1993) with a spontaneous natural mutation in *Btk,* and the other one is the engineered *Btk* knock-out (KO) mouse model (Hendriks et al., 1996; Khan et al., 1995). These mice have the B cell developmental block in later stages compared to humans and they usually have 50% of B cells in the periphery, and reduced levels of IgM and IgG3 with impaired response to some T-cell independent antigens (Conley et al., 2000). Perhaps a better model to study XLA is the *Btk/Tec* double KO model, where the phenotype is very similar to human XLA, as TEC is known to compensate for BTK in mice (Ellmeier et al., 2000).

There have been numerous successful preclinical studies that have been reported for XLA. The initial studies included *BTK* cDNA-expressing mice bred onto BTK deficient mice. These studies have shown to successfully restore the phenotype, especially when the cDNA sequence is optimized with important regulatory regions. The follow-up studies involved the use of retroviral and lentiviral vectors for the transduction of stem cells and transplanting

them back to the BTK deficient mice. These studies also confirmed that the optimization of *BTK* cDNA sequence results in better rescue of the phenotype, including in the *Btk/Tec* double KO mouse model (Berglöf et al., 2013; Hendriks et al., 2011). However, to date none of these studies have made it to the clinic. So alternative curative therapy options are still needed and this thesis is mainly addressing the non-viral strategies for the treatment of XLA.

1.2.1.1.1 BTK

BTK is a non-receptor cytoplasmic kinase and it is a member of TEC kinase family that includes: TEC, ITK, RLK/TXK and BMX, which are expressed in hematopoietic cells (Smith et al., 2001). BTK is expressed in myeloid cells, platelets and in B cells except the mature plasma cells (de Weers et al., 1993; Nisitani et al., 2000; Smith et al., 1994) and the inactivation of BTK seems to predominantly affect B cells (Mohamed et al., 2009). BTK, like other TEC kinase family members contain, from the N terminus, a pleckstrin homology (PH), Tec homology (TH), Src homology 3 (SH3), SH2, and the catalytic kinase domain (Mohamed et al., 2009). These domains are important for downstream signaling and the activation of BTK (Smith et al., 2001). When the B cell receptor (BCR) is activated, BTK is moved closer to the plasma membrane and another kinase family called, SRC family kinases, phosphorylates BTK at its Y551 position. Upon this, BTK is auto-phosphorylated at position Y223 in its SH3 domain. This then activates a signaling cascade that involves the phosphorylation of phospholipase Cγ2 (PLCγ2) and the subsequent activation of downstream elements like the calcium flux and NF-κB which are important for the proliferation, differentiation and the survival of B cells (Hendriks et al., 2011; Mohamed et al., 2009).

1.2.1.1.2 Mutations in the *BTK* gene

BTK is expressed from a 37.5 kb gene with 19 exons and more than 800 mutations have been reported for the gene (Valiaho et al., 2006). Typical mutations that account for more than 90% involve amino acid substitutions, splice site defects, premature stop codons and frame-shifts caused by insertions or deletions. The remaining mutations are large deletions, duplications, inversions or retro-transposon insertions (Conley et al., 2009). There is a huge diversity of mutations in *BTK* and no single change accounts for more than 3-6% of the mutations. The mutations are scattered throughout the gene except the SH3 domain where no missense mutations have been reported so far (Bestas et al., 2015; Lindvall et al., 2005). Moreover, the types of mutations might affect the disease severity as the mutations in less conserved regions such as amino acid substitutions in non-invariant sites or splicing defects, might result in partial production of BTK and cause milder XLA (Conley et al., 2009; Lindvall et al., 2005). All the known *BTK* mutations are gathered in the database called BTKbase (http://structure.bmc.lu.se/idbase/BTKbase/) (Valiaho et al., 2006).

A pronounced group (approx.13%) of XLA mutations involve splice site mutations and they have been reported by numerous studies (Bestas et al., 2015). These mutations can be in invariant splice sites as well as in intronic or exonic regions. They usually result in exon exclusion or the activation of cryptic splice sites, which might cause frame-shifts, usually resulting in NMD (Bestas et al., 2015). Two of the papers in this thesis are investigating the

possibility to manipulate splice site mutations in XLA. Paper II is based on a mutation in intron 4 (A to T) of *BTK* that creates a cryptic 3' ss. This results in inclusion of a cryptic exon, which creates a PTC and possibly leads to NMD. Paper III is based on several core-splice site mutations in the proximity of *BTK* exons 16 and 17 that are causing exon exclusion or cryptic splicing (see Results and Discussion).

1.2.1.2 Duchenne Muscular Dystrophy (DMD)

Duchenne muscular dystrophy (DMD) is an X-linked recessive fatal muscle-wasting disorder. It is caused by the deletions, point mutations and duplications in the *DMD* gene (Muntoni et al., 2003). Most of the mutations generate out-of-frame transcript that in the end leads to premature degradation of the RNA, resulting in the lack of dystrophin which is expressed in all muscle cells (skeletal, smooth and cardiac). The protein dystrophin (Hoffman et al., 1987) has an important role to connect the intracellular cytoskeleton to the extracellular matrix so the patients lacking it have muscle degeneration, which leads to cardiac and respiratory complications (Muntoni and Wood, 2011). There is a milder form of this disease called the Becker muscular dystrophy (BMD), which is also caused by the deletion and mutations in the *DMD* gene. BMD patients still retain in-frame transcripts that result in a truncated but still partially functional dystrophin (Koenig et al., 1989). Currently, there is a lot of research going on for the treatment of DMD with stem cell transplantation, delivery of coding gene with viral vectors, small molecules as well as gene editing (Long et al., 2014; Wood et al., 2010). However perhaps the most promising method is oligonucleotide therapy (see section 1.4.2.2.3), which is based on the information taken from the BMD patients, that dystrophin can handle deletions in its central repeat region while still maintaining its crucial function. Most of the deletions in *DMD* are observed between exons 45-55 **(Figure 4F)** and skipping of different parts has been studied. Exon 51 skipping that results in an in-frame transcript is the most extensively studied, and covers 13% of the DMD patients (Aartsma-Rus, 2012; Aartsma-Rus and van Ommen, 2007). The majority of the pre-clinical work in DMD has been done with the mdx mouse model. This mouse has a spontaneous mutation in exon 23 that creates a PTC (Sicinski et al., 1989), and skipping exon 23 restores the reading frame producing a partially functional protein. Since then, many preclinical and clinical trials have been conducted for DMD and currently two oligonucleotide-based candidate drugs are under investigation (McGreevy et al., 2015; Muntoni and Wood, 2011; Veltrop and Aartsma-Rus, 2014).

Another gene, which has also been studied in the context of DMD, is myostatin (*Mstn*). Myostatin inhibits muscle growth through the activation of activin receptor (AcvR2b) and loss of myostatin led to increased muscle mass and strength in the mdx mouse model (Kang et al., 2011; Lee, 2004; McPherron et al., 1997). Since then several strategies have been used to block myostatin via antibodies (Wagner et al., 2008) and inhibitory peptides (Bogdanovich et al., 2005) as well as gene silencing (Roberts et al., 2012). Moreover, a dual approach has been reported by suppressing myostatin and enhancing dystrophin expression via adeno-associated viruses, improving muscle function in mdx mice (Dumonceaux et al., 2010). Later on, dual oligonucleotide therapeutic approaches have also been applied in order to block

myostatin expression and produce in-frame dystrophin in mdx mouse model, showing beneficial effect (Kemaladewi et al., 2011; Malerba et al., 2012). In this thesis, a similar approach has been applied with a novel synthesis method in order to target *Mstn* and *Dmd* at the same time (Paper I, see Results and Discussion).

1.3 Gene therapy

Classical gene therapy, in its simplest form, is the introduction of nucleic acid material that codes for the correct gene in order to prevent a pathological condition. For this approach, viral or non-viral vectors can be utilized for the delivery.

In 1990, Dr. Steven A. Rosenberg performed the first approved gene therapy trial, where he used a marker gene to track lymphocytes in cancer patients (Rosenberg et al., 1990). Following this attempt, Dr. R. Michael Blaese was the first to conduct a gene therapy trial using a therapeutic transgene for adenosine deaminase deficiency (ADA-SCID) patients, yet the results were not satisfactory (Wirth et al., 2013).

The field faced a downfall when the gene therapy trial performed in 1999 resulted in the death of the patient Jesse Gelsinger, due to virus-related side effects. Following this in 2000, the field had an almost exciting turn when three children with an immunodeficiency disease were successfully treated with hematopoietic stem cell transduction via the Moloney murine leukemia virus (MLV) vector that carried the correct transgene. However, as some other treated children developed leukemia-like disorder, the need for understanding and optimizing the viral vector-host interaction became the crucial goal (Thomas et al., 2003).

Currently, 70% of the gene therapy vectors under investigation are the viral vectors and the most studied ones are the integrating (gammaretrovirus and lentivirus) and the episomal viruses (herpes simplex virus, adenovirus and adeno-associated virus) (Thomas et al., 2003; Verma and Weitzman, 2005). The crucial intrinsic problems of the viral vectors are immune response, hurdles with the targeted delivery and the insertional mutagenesis of viral genes, and there are numerous studies ongoing to overcome these problems (Kay, 2011). However, promising clinical results have been obtained by viral gene delivery in several diseases such as; β-thalassemia, X-linked severe combined immunodeficiency (SCID-X1), ADA-SCID and Wiskott-Aldrich syndrome (Wirth et al., 2013). Moreover, in 2012, Glybera became the first European Union approved gene therapy medicine. It is an adeno-associated viral vector for the treatment of severe lipoprotein lipase deficiency, and it is accepted as orphan drug designation both in EU and US. According to the Journal of Gene Medicine database (http://www.abedia.com/wiley/index.html), adenoviruses and the retroviruses are the most common vectors that are used for gene delivery, and the most investigated disease is cancer.

On the other hand, non-viral gene therapy is an alternative, less risky option, as these medicines do not maintain viral components, are able to handle larger transgenes and are less prone to activate any pre-existing immune response. Traditional non-viral vectors are mainly plasmid DNA and the associated polymers or lipid particles that are used for delivery. However, with the increasing understanding of gene regulation, nucleic acid materials such as small interfering RNAs (siRNAs), microRNAs (miRNAs) and antisense oligonucleotides

(AONs) that can interfere with gene expression by binding to the mRNA are also included in this group (see section 1.4.2). In addition, delivery of exogenous mRNA and the modulation of splicing by the expression of non-coding RNAs are also considered in this category (Kay, 2011; Yin et al., 2014). Moreover, recent genome editing technologies such as zinc-finger proteins (ZFP), transcription activator-like effectors (TALEs) and CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat-CRISPR-associated), are gaining a lot of attention in the gene therapy field (Maggio and Goncalves, 2015) and are already under pre-clinical and clinical investigation.

1.3.1 Delivery issue of non-viral vectors

While viruses have evolved to enter into the mammalian cells, non-viral vectors are far less efficient when it comes to delivery. Especially, one of the biggest hurdles is the in vivo delivery. This brings other barriers such as degradation by nucleases, renal clearance, extracellular matrix, cell membrane, endosomes and eventually the nuclear membrane (Wang et al., 2013).

Naked DNA has been delivered via intravenous injection as well as physical methods such as hydrodynamic injection, gene gun, electroporation or ultrasound mediated techniques. The underlying mechanism of the physical methods is to make the cell membrane permeable via physical forces. For example, electroporation causes temporary destabilization in the cell membrane by the controlled exposure to an electric field, which allows the genetic material to enter the cell (Ibraheem et al., 2014). However, these methods are not very applicable when it comes to humans, so optimization of chemical methods emerged as an alternative. These chemical methods involve the use of synthetic vehicles including lipid-based systems (lipoplexes), polymers (polyplexes), biodegradable polymers, peptides (Martin and Rice, 2007) and inorganic materials, which are believed to enter the cells via endocytotic pathways (Khalil et al., 2006) **(Figure 5)**. The mechanism of chemical vehicles is mainly to have electrostatic interaction between their cationic moieties and the anionic phosphate of the DNA cargo (Ibraheem et al., 2014). Modification of these vehicles with pH sensitive moieties for endosomal escape, nuclear localization signal (NLS) for nuclear uptake and ligand attachment for cell-specific delivery, are common ways to increase their efficiency. In addition hydrophilic polymers such as polyethylene glycol moiety (PEG) are used in order to avoid toxicity, renal clearance and the recognition by the phagocyte system due to unspecific interaction and aggregation (Viola et al., 2010; Wang et al., 2013). More advanced nano-particles, which combine several of these properties, are undergoing clinical trials for the delivery of mainly siRNA and plasmid DNA (Yin et al., 2014).

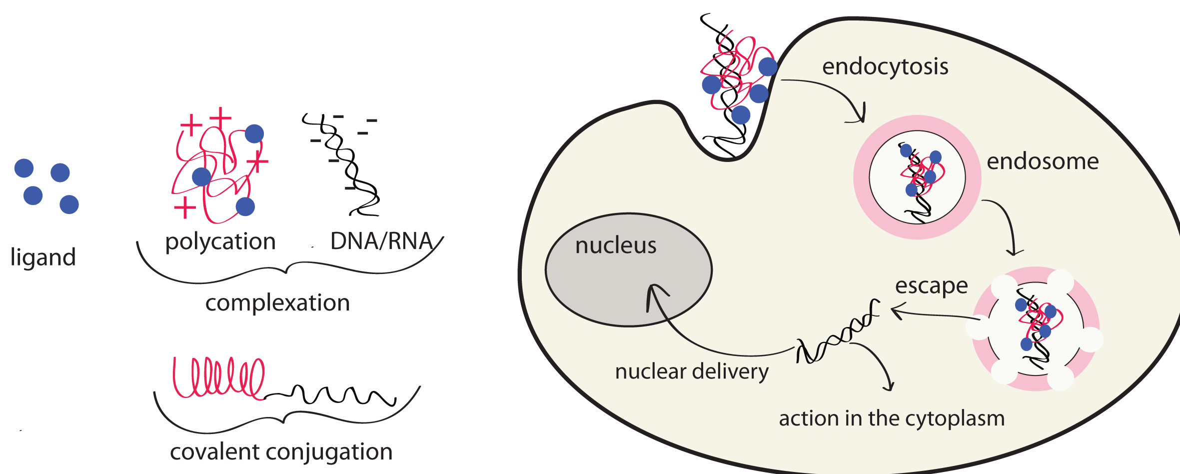


Figure 5. A simple scheme for the non-viral delivery approach. Cationic vehicle is either in complexation with the DNA/RNA molecule or in covalent conjugation (e.g. cell-penetrating peptides, see section 1.4.1.1). Upon internalization via endocytosis, the therapeutic has to escape from the endosomes in order to be active, either by shuttling to the nucleus or in the cytoplasm, depending on the purpose. Ligands that can be used for targeted delivery are depicted in blue.

The encapsulation of the non-viral vector is also crucial for the biological half-life of the vector, since they are prone to degradation by serum endonucleases as well as renal clearance. On the other hand, release of the DNA vector from the carrier is also an important parameter for efficient gene expression. It has been proposed that the release of DNA vectors from polymer-based particles is slower compared to lipid-based particles as polymer-DNA complexes were found in the nucleus, possibly hampering the expression (Lam and Dean, 2010; Yin et al., 2014).

Moreover, expression levels of DNA vectors diminish over time. There are ongoing studies for enhancing the expression via optimization of the regulatory sequences (Yin et al., 2014) or via minicircle DNA plasmids that are devoid of bacterial elements in their backbone (Kay et al., 2010). Although DNA vectors are not integrating and if so with very low levels, exploiting the transposon systems such as *Sleeping Beauty* and *PiggyBac* are under clinical investigation for therapeutic gene transfer into the primary human cells (Aronovich et al., 2011; Singh et al., 2014).

1.4 Antisense oligonucleotide therapy

In parallel with the evolution of gene therapy, another field emerged as possible therapeutics for the modulation of gene expression, antisense oligonucleotide therapeutics. The birth of antisense field can be attributed to the work of Zamecnik and Stephenson, where they reported the inhibition of virus replication in cell culture by using single-stranded antisense DNA-based oligonucleotides (Stephenson and Zamecnik, 1978). Antisense oligonucleotides (AONs) are short stretches (8-50 nt) of DNA/RNA molecules that can bind to their complementary target RNA through Watson-Crick base pairing. Moreover, the implementation of solid-phase synthesis methods also accelerated the antisense field immensely, as now many variants are produced using this technique (Matteucci and Caruthers, 1992).

1.4.1 Antisense Oligonucleotide Chemistry

One can imagine that the target of AONs can be within short sequences as well as in a long stretch of an RNA molecule, like the pre-mRNA. Another layer of complexity comes with the secondary structure of the target, since RNAs are usually in complex with proteins and form dynamic structures. So the AONs should find their target with high fidelity and this has been, and still is, one of the key issues in the field besides the delivery issue. During the last decade, chemists have studied numerous modifications for AONs. The underlying reasons for these modifications are mainly increasing biological half-life, increasing target affinity, increasing cellular uptake and not inducing toxicity or immune response.

Unmodified DNA/RNA oligonucleotides are inherently unstable and they are easily cleared away from the body by kidneys, via binding to the proteins in plasma. Modifications in the sugar ring and the phosphodiester backbone are the most studied positions for increasing the stability of AONs. One of the earliest and the most widely used modifications are the phosphorothioate (PS)-containing oligonucleotides, where one of the non-bridging phosphate oxygen atoms is replaced with a sulfur atom **(Figure 6)**. This modification increases the stability of oligonucleotides against nucleases (Stein et al., 1988). Moreover, PS-modified AONs are reported to bind to plasma proteins, which increases their circulation time since the kidney cannot clear them away so easily. On the other hand, this binding tendency can also induce toxicity due to unspecific binding to proteins that might be important for the cell function (Geary, 2009; Levin, 1999). The first FDA-approved AON is Vitravene (Fomivirsen), which is a 21-mer DNA-based PS AON for the treatment of cytomegalovirus retinitis (Jabs and Griffiths, 2002).

Replacement of the sugar-phosphate backbone with an entirely different chemistry is another strategy in the AON field. One of these modifications is phosphorodiamidate morpholino oligomer (PMO). PMOs have a morpholine ring as a replacement for the ribose and a phosphorodiamidate linkage instead of the phosphodiester backbone, which makes them electrochemically neutral (Summerton, 1999) **(Figure 6)**. On one hand the neutral nature of PMOs gives them high renal clearance, yet on the other hand high doses of PMO can be tolerated in vivo (Wu et al., 2011). PMOs have been efficiently used for the manipulation of splicing (Alter et al., 2006; Sazani et al., 2002; Wu et al., 2008) as well as translation arrest in viral infections (Swenson et al., 2009). Moreover, also owing to their neutral nature; they have been widely used in conjugation with cell-penetrating peptides (CPPs) (El Andaloussi et al., 2012); CPPs have the ability to translocate through the cell membrane (see detailed description in the next section). A PMO-based AON Eteplirsen (Sarepta) is currently under clinical trial for the treatment of Duchenne muscular dystrophy (DMD) for exon skipping (Lu et al., 2014). Another modification is the peptide nucleic acid (PNA), which is prepared by replacing the sugar-phosphate backbone with N-(2-aminoethyl) glycine linkage and nucleobases attached through methylene carbonyl linkage to the glycine amino group (Nielsen et al., 1991) **(Figure 6)**. PNAs are uncharged and have high affinity to DNA and RNA as well as increased stability. PNAs have been used for translation arrest (Nulf and Corey, 2004) and splice manipulation studies (Sazani et al., 2002; Siwkowski et al., 2004). The big drawback is that PNAs cannot cross the cell membrane on their own, and for this

reason they have been mostly conjugated with peptides to increase their cellular uptake (Albertshofer et al., 2005; Maier et al., 2006).

The most widely studied modifications are in the 2'-position of the sugar moiety group, 2' -*O*-methyl (2'-OMe) and the 2'-*O*-methoxyethyl (MOE) **(Figure 6)**. MOE AONs have increased stability since the bulky 2'-substituent is close to the 3'-phosphate, in theory protecting it from degradation. Both modifications are usually used together with the PS modification. The drawback for the 2'-modifications is that they are not compatible with RNase H mechanism, which is why the AONs are designed as "gapmers" where the central part are DNA-PS but the flanking regions are modified for the increased stability. Gapmers are short oligonucleotides that can bind to target RNA and initiate degradation via RNase H enzyme (see section 1.4.2.1.1). 2'-OMe modifications are well tolerated in siRNAs as they have been shown to have effect in pre-clinical in vivo studies (Zimmermann et al., 2006) and they have been reported to reduce the off-target effects via careful positioning (Jackson et al., 2003). Moreover, 2'-OMe-PS modifications have also been widely investigated in the splicing field, where the aim is to manipulate the faulty splicing due to mutations (see section 1.4.2.2.3). A candidate 2'-OMe-PS-based AON PRO051/Drisapersen (Prosensa) made it to the Phase III clinical trial for the treatment of DMD. However, later on it was announced that the outcome did not meet required expectations for improvement (Lu et al., 2014). MOE modification has also proven to have a lot of potential within the AON field. It is very stable against nucleases and it is thought to reduce unspecific binding to proteins in vivo. Moreover, MOE is reported to induce C3'-*endo* conformation, which forces the oligonucleotide into an A-helical geometry, known to increase binding to RNA (Teplova et al., 1999). A MOE-based AON ISIS-SMN_{rx} (ISIS), is under clinical trial for the enhancement of exon inclusion in spinal muscular atrophy (SMA) (Rigo et al., 2012). In addition, a MOE-based gapmer KYNAMRO® (ISIS) has been approved by Food and Drug Administration (FDA) for the treatment of familial hypercholesterolemia.

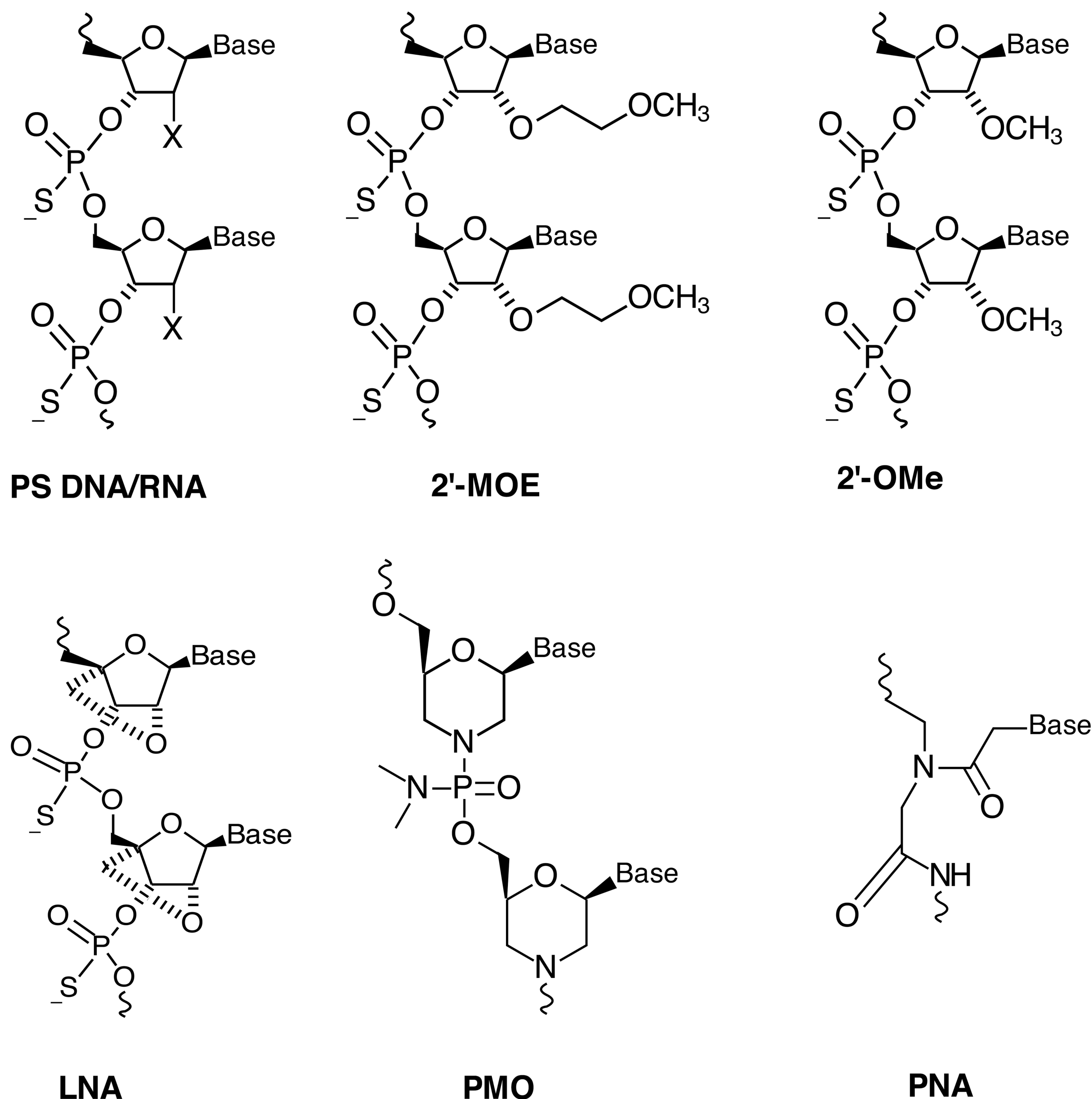


Figure 6. Antisense oligonucleotide chemistries. Phosphorothioate (PS), 2'-*O*-methoxyethyl (MOE), 2'-*O*-methyl (2'-OMe), Locked Nucleic Acid (LNA), phosphorodiamidate morpholino oligomer (PMO) and peptide nucleic acid (PNA). X is "H" for DNA and "OH" for RNA.

A very high target affinity is achieved by the Locked Nucleic Acid (LNA) modification (Lundin et al., 2013). This is also a modification in the sugar moiety where the 2'-substituent is locked to the 4'-C atom **(Figure 6)**. This modification forces the oligonucleotide to be in the A-helical form, dramatically increasing the binding affinity to RNA (Nielsen and Spielmann, 2005; Petersen et al., 2002). In the AON field, the LNA modification has been investigated for splice manipulation, miRNA targeting, gapmers and DNA targeting (anti-gene) approaches (Lundin et al., 2013) (see section 1.4.2). It has been reported to increase serum stability (Kurreck et al., 2002; Wahlestedt et al., 2000), slower renal clearance from the body (Roberts et al., 2006) and also has proven to have an in vivo effect (Elmen et al., 2008; Lanford et al., 2010; Lindholm et al., 2012; Seth et al., 2009; Straarup et al., 2010). However, toxicity and possibility of off-target effects have also been reported in some studies (Guterstam et al., 2008; Swayze et al., 2007).

1.4.1.1 Cell-penetrating peptides (CPPs)

For the purpose of this thesis, this section will explain the use of cell-penetrating peptides for the delivery of AONs, especially for the modulation of splicing. The cellular barriers that are mentioned (see section 1.3.1) also apply to the AON delivery. In most cases AONs are delivered to the cells by cationic lipids, dendrimers or electroporation as well as by naked injection in vivo. Moreover, for the purpose of in vivo delivery, cationic polymers and peptides are also under investigation (Lehto and Wagner, 2014), and one of the most used are the cell-penetrating peptides (CPP) (Boisguerin et al., 2015; Derossi et al., 1994).

The concept of cell-penetrating peptides emerged when it was discovered that a protein in drosophila could translocate through the cellular membrane (Derossi et al., 1994). Following this, virologists found a cationic peptide that could enable the transport of macromolecules through the cell membrane (Roebuck et al., 1997). Cell-penetrating peptides have the ability to rapidly translocate through the cell membrane and deliver their cargo. The common properties of CPPs are, (i) they are 5-40 amino acids in length; (ii) they carry net positive charge; (iii) they can have natural or non-natural amino acids; (iv) they can carry their cargo via in covalent conjugation or in non-conjugated form (El Andaloussi et al., 2012) **(Figure5)**. The properties of CPPs might differ in hydrophobicity and secondary structure, which in the end codes for their function. The internalization of CPPs is mainly associated with endocytotic pathways or energy-independent translocation and it is thought that depending on the cell type, CPP sequence and concentration, different routes might be used (Boisguerin et al., 2015). CPPs have been reported to deliver their cargo to different types of organs, including challenging ones such as the brain and the heart (Du et al., 2011; El Andaloussi et al., 2012; Yin et al., 2011).

An important issue here is that only the charge neutral AONs, PNAs and PMOs, can be used in covalent conjugation to the positively charged CPPs, and covalent conjugation of PMOs is more common in the splicing field (Lebleu et al., 2008). Another important note is that polycationic CPPs were found to be entrapped to a larger extent in the endosomes whereas amphipathic variants were more efficient in having an effect (El-Andaloussi et al., 2006). This difference is thought to be due to the fact that the amphipathic CPPs can interact more strongly with cellular membranes, and they might hence be disrupting the endosomes and releasing the cargo. Amphipathic CPPs can have both hydrophobic and electrostatic interactions with their cargo. Moreover, addition of hydrophobic components, the distribution of the charge and the secondary structure of the CPPs seem to have an important role in their efficiency, affecting the final stability and the endosomal escape capability (El Andaloussi et al., 2012; Lehto et al., 2014).

CPPs have been tested in splicing diseases such as, β-thalassemia, spinal muscular atrophy (SMA) and mostly in Duchenne muscular dystrophy (DMD). One of the most studied CPP variants, especially in DMD, is the arginine rich B-peptide which was reported to be efficient in preclinical studies when conjugated to a functional PMO (Du et al., 2011; Jearawiriyapaisarn et al., 2008; Wu et al., 2008). Another efficient and more optimized group of CPPs are the PMO/PNA internalizing peptides (Pips). These peptides have a hydrophobic

core, arginine rich flanking regions and spacer elements. Pips have been shown to be more efficient for targeting the heart compared to the B-peptide and new variants of Pips are under investigation (Betts et al., 2015; Lehto et al., 2014; Yin et al., 2011). CPP-conjugates have not made to clinical trials yet, since their optimization is still under investigation. Pre-clinical studies show that high doses of PMO can be tolerated but achieving an effect in challenging organs is a problem whereas CPPs can enhance the activity for more effective and targeted delivery.

1.4.1.2 Considerations about antisense oligonucleotide uptake and biodistribution

Considering the different chemistries explained above **(Figure 6)** and their properties, it is not surprising that they will have different properties once injected in vivo (Geary et al., 2015). Different biodistribution studies of the AONs have been performed and gapmers (see section 1.4.2.1.1) are one of the mostly studied AONs on this issue. The main routes of injection for AONs are intravenous (IV) infusion or subcutaneous (SC) injection whereas more localized intrathecal administrations have also been performed. Once injected, PS-modified AONs rapidly relocate to tissues and they can circulate in the body around 2-4 weeks (Geary et al., 2015). AONs that have PS modification bind to plasma proteins extensively and albumin is the most common protein that they bind to. Since the albumin binding is not very strong, AONs can still be distributed to tissues without being cleared away, thus this balance seems to be crucial (Graham et al., 2007). On the other hand, due to their uncharged nature, PMOs are cleared away from the body faster (Amantana and Iversen, 2005) and they primarily localize in the kidneys. Although AONs mainly localize in the liver and kidneys, a recent study reported AON activity in a wide range of tissues and organs when injected systemically (Hung et al., 2013). There is also another consideration about the injection dose and administration route, depending on the target organ, as a single high dose or gradual administration of lower dose might have different results (Geary et al., 2009).

Another issue concerning the smaller scale of the events is that there is no definite understanding about how the AONs are internalized and shuttled inside the cells **(Figure 5)**. In general, the mechanism of internalization is not yet described but believed to be one of several pathways of endocytosis such as via clathrin-coated pits, caveolar pathway, clathrin/caveolin-independent pathways or macropinocytosis. The entry through these mechanisms starts intracellular trafficking involving early/late endosomes, lysosomes and the Golgi apparatus. During all these trafficking the pH, content of the molecules such as the proteins and lipids are highly dynamic (Juliano et al., 2014). So during all these dissociation and association events of the intracellular membranes, AONs might be escaping and one can imagine that AONs are prone to interacting with different kinds of cellular components during these processes (Liang et al., 2014). The common tactic in the field is to conjugate ligands to the AONs that the cells would recognize and initiate internalization pathway. These ligands might be in direct conjugation with AONs or in conjugation with their polymeric/lipid-based carriers. The key issues in this approach are the ligand efficiency when coupled to AONs/carriers as well as the side effects to the cells (Juliano et al., 2014). Besides the investigation of non-viral vector/vehicle entry to the cells, there is also the concept of

"naked uptake/gymnosis". Recently, there have been interesting reports, showing that cells can internalize AONs without the aid of any transfection reagent (vehicle), authors named the concept as "gymnosis" (Stein et al., 2010; Zhang et al., 2011). An important parameter here is that the internalization and shuttling of these naked AONs might change depending on the chemistry as well as the target cell type (Koller et al., 2011). Although some cell surface receptors have been suggested to mediate naked AON uptake, such as scavenger receptors (Limmon et al., 2008; Pearson et al., 1993), there is no consensus on this issue yet. Interestingly, scavenger receptors have also been shown to have a role in peptide-mediated uptake of AONs (Ezzat et al., 2012). Overall, understanding the AON internalization and shuttling inside the cells would help designing better therapeutics, and this is still waiting to be resolved.

1.4.2 Antisense Oligonucleotide Mechanisms

AON mechanisms can be classified as occupancy-induced destabilization and occupancy-only mechanisms. Occupancy-induced destabilization is the degradation of RNA (siRNAs, RNase H, Ribozymes, DNAzymes) whereas occupancy-only mechanism is the alteration of splicing, translational silencing, miRNA-mediated gene silencing and the disruption of RNA structure (Crooke, 2008).

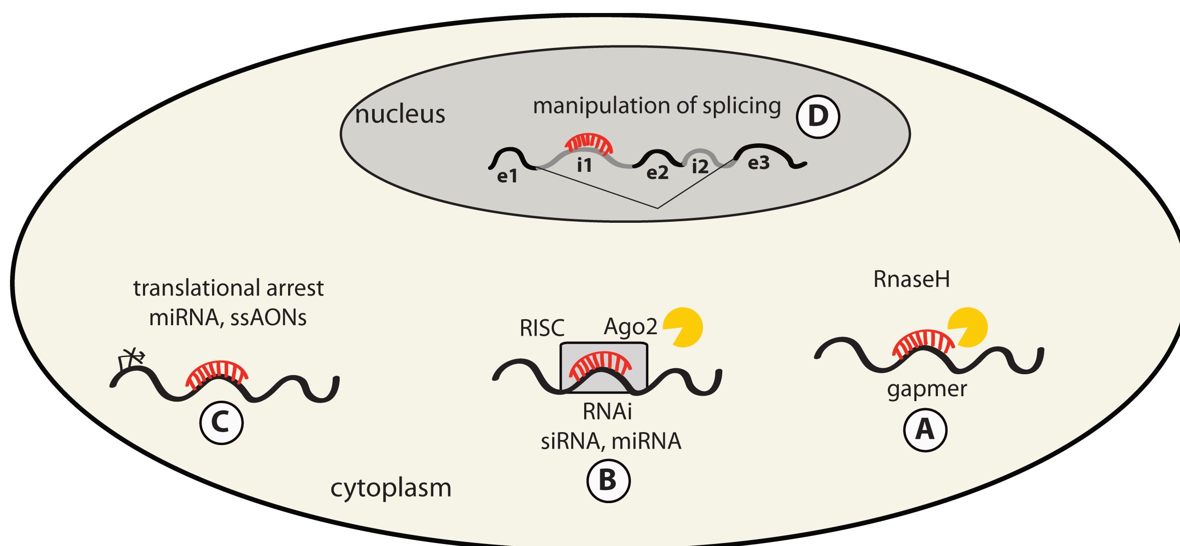


Figure 7. A summary for the main action routes of antisense oligonucleotides. A. AONs (gapmers) bind to the mRNA and activate RnaseH degradation mechanism. **B.** Small interfering RNAs (siRNAs) activate RNA-induced silencing complex (RISC), degrading the target RNA. MicroRNAs (miRs) can also induce RISC. **C.** Single-stranded AONs (ssAONs) either by altering the RNA structure or re-directing the polyadenylation, can induce RNA degradation. MiRs bind to the 3'UTR of the target RNA and activate RNA degradation. **D.** Splice-switching oligonucleotides (SSOs) can bind to the pre-mRNA and manipulate the splicing mechanism, e:exons and i:introns. AONs are depicted in red.

1.4.2.1 Occupancy-induced destabilization mechanisms of AONs

1.4.2.1.1 RNase H and gapmers.

One of the most robust degradation mechanisms in the cell is the RNase H pathway in which DNA-RNA hybrids are hydrolyzed. Two classes of this enzyme with different cofactor requirements have been found in mammalian cells, RNase H1 and RNase H2 (Busen, 1980; Eder and Walder, 1991; Frank et al., 1994). RNase H proteins are known to have a role during DNA replication and resolving RNA loops during transcription (Cerritelli and Crouch, 2009).

Studies revealed that RNase H1 is the enzyme responsible for DNA antisense oligonucleotide/RNA hybrid degradation mechanism (Wu et al., 2004). It has been found that RNase H1 cleaves the DNA-RNA hybrid preferentially 7-10 nucleotides from the 3' DNA/5' RNA terminus and modification of the nucleotides from 3' of DNA substrate can hamper the enzyme activity (Lima et al., 2003). Moreover, follow-up structural studies for the catalytic activity revealed that RNase H1 preferentially binds to the sugar pucker with eastern O'_4-*endo* conformation, which occurs when DNA binds to RNA (Lima et al., 2004). These studies thus clarified some important requirement for RNase H1 function that paved the way for designing antisense therapeutics. The preferred design of the DNA oligonucleotide substrate are determined to be: i) a conformationally flexible sugar producing an O'_4-*endo* pucker when hybridized to RNA, ii) no sterically bulky 2'-substituents, iii) a conformationally rigid phosphate backbone (Crooke, 2008).

The understanding of the RNase H1 mechanism created the concept of "gapmers" that are widely used for RNA degradation in order to knock down a gene **(Figure 7A)**. Gapmers are typically single-stranded AONs, that are composed of 8-10 PS-modified DNA nucleotides in the center and flanking modified nucleotides on both sides (Monia et al., 1993). Several modifications have been investigated, and the most efficient combination turned out be the 2'-MOE modifications in the flanking regions with a stretch of PS DNA nucleotides at the center. Recently, FDA has approved such a gapmer with 2'-MOE modification for the treatment of familial hypercholesterolemia, targeting apolipoprotein B (*APOB*). LNA modification has also been exploited for gapmers, as it turned out to be compatible with RNase H activity. In addition, LNA has been reported to be efficient in vivo (Straarup et al., 2010), although risk of hepatotoxicity has also been mentioned (Swayze et al., 2007) Moreover, a recently completed Phase-I clinical trial reported to have promising results. The study involves a gapmer (AZD9150) that targets signal transducer and activator of transcription 3 (*STAT3*) for B cell lymphoma patients.

1.4.2.1.2 Small interfering RNAs (siRNAs)

RNA interference (RNAi) was first described in Caenorhabditis elegans when Fire and Mello injected double-stranded RNA (dsRNA) molecules that sequence specifically down-regulated the target mRNA (Fire et al., 1998), which later on led them gain the Nobel Prize in 2006. In mammalian cells, precursor dsRNAs are cleaved by the RNaseIII endonuclease Dicer to produce siRNA that usually has a 2nt overhang at the 3' end. Later on, Dicer complexes with

TAR-RNA binding protein (TRPBP) and loads the siRNA to the RNA-induced silencing complex (RISC). RISC contains the "slicing" protein Argonaute 2 (Ago2) which cleaves the target mRNA between the bases 10 and 11 from the 5'-end of the antisense siRNA. When siRNA is loaded onto the RISC complex, Ago2 cleaves the "passenger" strand and leaves the "guide" strand for binding to the target mRNA (Aagaard and Rossi, 2007; Gavrilov and Saltzman, 2012). Thermodynamic data reveals that Ago2 selects the guide strand with the less stable 5' end (Schwarz et al., 2003).

Since double stranded RNAs, when introduced into the cells, induce apoptosis as a defense mechanism (Garcia et al., 2007), exogenous delivery of already processed siRNAs became an option for gene silencing. These synthetic siRNAs are double stranded 21-23 nt long oligonucleotides that can be exogenously administered to cells in order to initiate the RNAi pathway without the Dicer cleavage **(Figure 7B)**. The first demonstration of this approach was shown in 2001 (Elbashir et al., 2001) and since then it has become a wide research field.

The design of siRNAs holds a crucial importance for its activity and some important parameters to consider are structural features, chemical modifications, target availability and thermodynamic end stability (Gavrilov and Saltzman, 2012). The major drawbacks of siRNAs are the delivery issue and the stability problem against nucleases and numerous chemical modifications are under investigation to overcome these problems (Bramsen and Kjems, 2012; Rettig and Behlke, 2012). The most widely studied modification is for the 2'-*O* position of the sugar and one of the most successful modifications is 2'-fluoro RNA (2'-F) that is more tolerated compared to the bulky 2'-MOE (Manoharan et al., 2011). Moreover PS modifications, albeit increasing stability, might cause cytotoxicity (Stessl et al., 2009). Another successful modification is to use 2'-OMe together with 2'-F RNA, and such a siRNA is currently under clinical trial by Alnylam Pharmaceuticals for the treatment of a neurodegenerative disease, transthyretin amyloidosis (Allerson et al., 2005).

Different delivery options have been tried for siRNAs such as cationic lipid-based particles, polymers (e.g. chitosan, PEI, dendrimers) with the addition of ligands for targeted delivery (e.g. aptamers, peptides, antibodies) and CPPs (Gavrilov and Saltzman, 2012). Recent reports show the successful delivery of lipid-based vehicle/siRNA complexes for hepatic silencing in non-human primates (Jayaraman et al., 2012). Furthermore, more preclinical and clinical studies are under investigation for metabolic and neurological disorders as well as cancer (Battistella and Marsden, 2015).

There are other crucial considerations for the application of siRNAs such as immune response and off-target effects (Jackson et al., 2003). It has been reported that certain sequence motifs, e.g. like "GU" rich sequences, are recognized by the TLR7 receptors and activate immune response (Hornung et al., 2005).

Another possible way to use the RNAi pathway is to use short hairpin RNAs (shRNAs), which are usually expressed from viral vectors. This approach is an alternative and more sustainable with long-term expression compared to the transient effect of synthetic siRNAs, although it also has drawbacks such as the saturation of the RNAi machinery in the cell

(Aagaard and Rossi, 2007; Grimm et al., 2006). Moreover, another approach called as single-stranded siRNAs (ss-siRNAs) is also shown to be effective in the RNAi pathway, which might reduce the risk of immune response (Lima et al., 2012; Yu et al., 2012).

An important note here is that although the RNAi pathway has been found to be active in the cytoplasm, it is also possible to use siRNAs for silencing gene expression in the nucleus via a mechanism called transcriptional gene silencing (TGS). Both siRNAs and microRNAs (mirRs) (see next section) are found to inhibit gene expression in the nucleus. Promoter associated RNAs (pRNAs) are thought to recruit Argonaute and induce repressive chromatin markers, though the mechanism needs further research (Rigo et al., 2014).

1.4.2.2 Occupancy-only mediated mechanisms of AONs

1.4.2.2.1 miRNA mimics and anti-miRNA AONs

MicroRNAs (miRs) are the endogenous components of the RNAi machinery. They are single stranded RNA molecules and typically are 21-25 nt long. They are initially expressed as primary micro-RNAs (pri-miRNAs) as 60-70 nt long molecules and processed by Drosha-DGCR8 to have a hairpin structure with 5'-phosphate group and 3'-two nucleotide overhang. Following this, they are exported to the cytoplasm and further processed by RNaseIII endonuclease Dicer. Similar to the RNAi pathway, one of the strands is loaded onto RISC for targeting mRNA. In contrast to siRNA, miRs have partial complementarity to the 3' UTR of mRNAs (3-8 nt) and their primary action is to repress translation **(Figure 7C)**, however they can also mediate RNA cleavage **(Figure 7B)** by recruiting other factors (Aagaard and Rossi, 2007; Bagga et al., 2005; Battistella and Marsden, 2015).

The mechanism of miRs can be mimicked by siRNAs by introducing mismatches in the guide strand and thus inducing translational arrest (Doench et al., 2003; Hu et al., 2010). On the other hand, AONs can be designed for blocking the activity of endogenous miRs, anti-miRNA AONs (AMOs), or block the binding sites of miRs. AMOs have been shown to work in preclinical studies (Elmen et al., 2008) and a DNA/LNA PS mixmer AMO Miravirsen, for hepatitis C virus (HCV) infection, has completed a phase-II clinical trial and investigations are still ongoing (Janssen et al., 2013). In addition, another LNA AON design as short as 8-mers, called tiny LNAs, have been shown to down-regulate the target miRNA in mice (Obad et al., 2011).

1.4.2.2.2 Translation arrest and RNA structure alteration

Other approaches were also reported for blocking translation and inducing RNA degradation. Single-stranded AONs can redirect the polyadenylation mechanism (Vickers et al., 2001), and siRNAs that are binding to the polyadenylation sites can induce degradation via an Ago-independent mechanism (Vickers and Crooke, 2012).

Another strategy by Goraczniak *et.al.* uses U1 adaptors for inducing RNA degradation (Goraczniak et al., 2009). In this work, the authors designed a bifunctional AON that is complementary to the 3'-UTR of pre-mRNA and also can bind to the 5'-end of U1 snRNA. By recruiting U1 snRNP to the site, polyadenylation is blocked and degradation of the target

is induced (Gunderson et al., 1998). In addition, the alteration of RNA secondary structure can prevent its expression as shown by AONs that can inhibit the expression of HIV via altering the transcript structure (Ivanova et al., 2007).

More complex mechanisms are also under investigation, including blocking ribosome assembly, targeting noncoding RNAs (ncRNAs) and the interference with the promoter associated RNAs (pRNAs) for the regulation of gene expression, yet more studies are needed for these approaches (Rigo et al., 2014).

1.4.2.2.3 Splice-switching oligonucleotide therapy for mis-splicing diseases

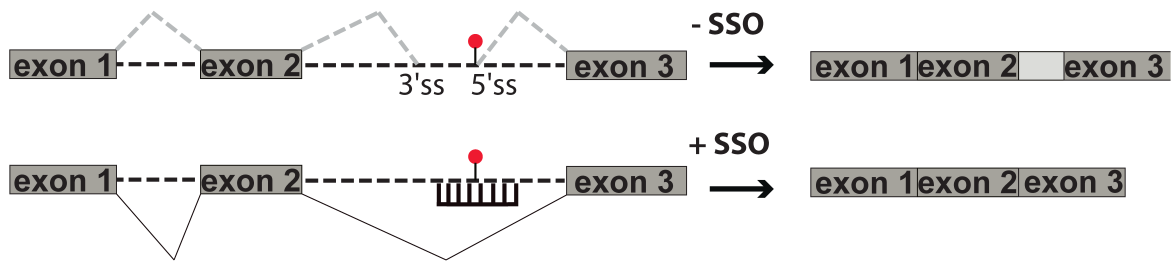
The first section detailed the mechanism of splicing, and how splicing is affected by mutations. Here I will discuss how to manipulate splicing in order to correct splicing defects, i.e. the so-called splice-switching oligonucleotide therapies.

Several splicing defects have been investigated in the field, such as cryptic/pseudo splice sites, mutated regulatory sequences and faulty alternative splicing (Hammond and Wood, 2011; Havens et al., 2013; Kole et al., 2012; Rigo et al., 2014). The main aim in all these studies is to use splice-switching oligonucleotides (SSOs) that bind to the pre-mRNA and manipulate splicing via steric hindrance or via the recruitment of the trans-splicing factors for splice site recognition.

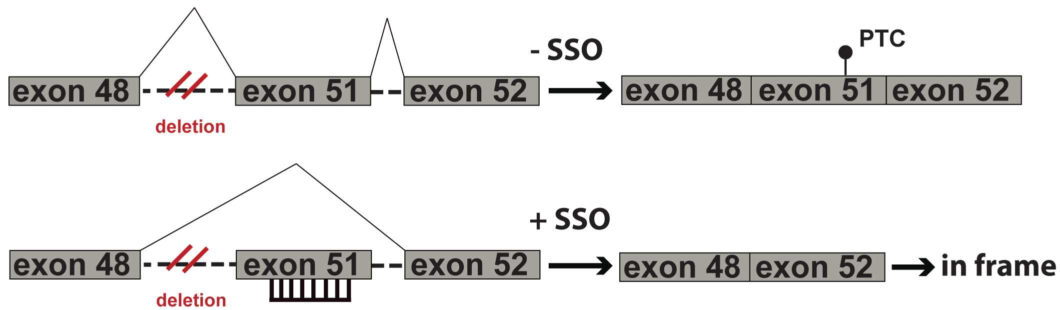
The first proof-of-concept was performed by the steric blocking of the cryptic splice site in intron 2 of the human β-*globin* gene, where the SSOs could restore the mRNA in a reporter cell line (Dominski and Kole, 1993) **(Figure 8A)**. Later on, this study was advanced by using CPP-PMO conjugates and showed the restoration of hemoglobin (1-5%) in the peripheral blood of a chimeric mouse model (IVS2–654) (Svasti et al., 2009).

The most investigated SSO chemistries are PMO and 2'-OMe PS and both of them have gone into clinical trials. For the clinical studies in DMD, SSOs were designed to induce exon 51 skipping as a treatment, which covers approximately 13% of the DMD patients **(Figure 8B)**. PMO and 2'-OMe PS-based SSOs have been investigated in pre-clinical (Alter et al., 2006; Mann et al., 2001) and two independent clinical trials with the AONs Eteplirsen (AVI-4658) and Drisapersen (PRO051) (Kinali et al., 2009; van Deutekom et al., 2007). Despite the promising results during the initial phase of the clinical trials, both SSOs were later on reported not to meet the clinical demand, but the results are still encouraging and investigations are ongoing (Cirak et al., 2011; Flanigan et al., 2014; Goemans et al., 2011; Mendell et al., 2013). Moreover, besides naked SSOs, cell-penetrating peptide (CPP) conjugated PMOs are under investigation in the mdx mouse model. Several studies reported encouraging results with even improvement in the cardiac function of the treated mice (Betts et al., 2012; Wu et al., 2008).

A) Cryptic splice site: β-*globin*



B) Exon exclusion: *DMD*



C) Exon inclusion: *SMN2*

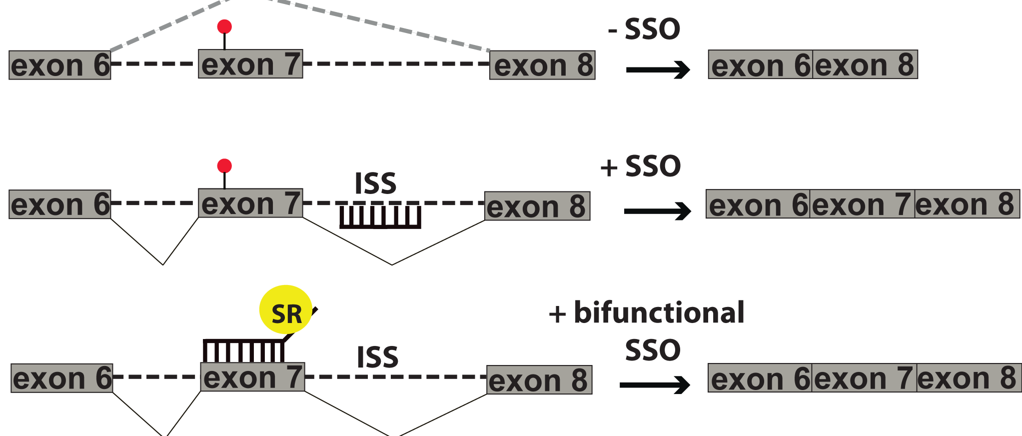


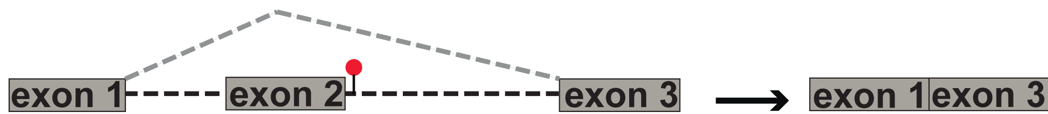
Figure 8. Methods for manipulation of splicing. A. Mutation in the intron 2 of the β-*globin* gene creates a 5'ss and activates 3' cryptic splice site, resulting in the inclusion of an intronic part. SSOs can sterically block the mutation and restore the splicing by generating transcripts without aberrant splicing. **B.** Deletion of the exons 49-50 generates an out-of-frame *DMD* transcript resulting in PTC. SSOs can rescue the splicing by skipping exon 51, which restores the reading frame and generates a partially functional protein. **C.** Mutation in the exon 7 of the *SMN2* gene results in exon 7 exclusion (in 90% of the transcripts) either by disrupting an enhancer element or by creating a silencer element. SSOs sterically blocking an ISS in intron 7 can restore the splicing and induce exon 7 inclusion. Another approach is to use bifunctional SSOs to enhance exon 7 inclusion in *SMN2* gene. These SSOs recruit trans-acting factors by their protein-binding tail and enhance exon 7 inclusion. Black pin represents PTC and red pin represents mutation. ISS: intronic splicing silencer

In contrast to exon skipping, SSOs can also be designed to enhance exon inclusion. An example of this concept is the spinal muscular atrophy (SMA). SSOs have been used to promote exon inclusion and restore the protein (Rigo et al., 2012) **(Figure 8C)**. Naked SSOs have been shown to function in this model. To date, the most successful SSO is MOE-based and it is sterically blocking a splicing silencer in intron 7 of the *SMN2* transcript (Singh et al., 2006). Moreover, when injected into the severe SMA mouse model, SSOs significantly improved survival of the mouse (Hua et al., 2010; Hua et al., 2011; Passini et al., 2011). Based on the encouraging pre-clinical and phase I trial results (Disterer et al., 2014), candidate SSO ISIS-SMNRx is currently in phase II clinical trial by Isis Pharmaceuticals.

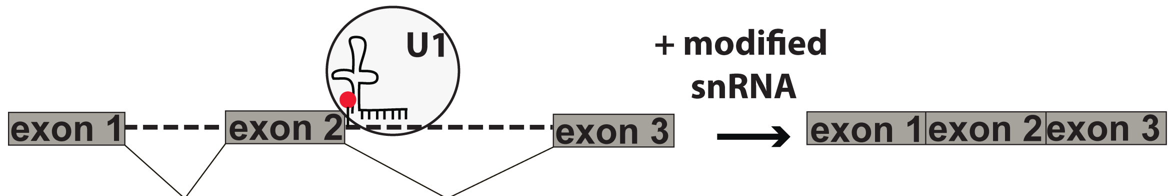
Moreover, SSOs called bifunctional oligonucleotides have also been applied. These oligonucleotides are typically 40-50 nt long. They have the ability to bind to their target sequence and recruit either a splicing enhancer or silencer protein factor via their free tail **(Figure 8C)**. Recruiting SR proteins to exon 7 of SMA patient fibroblast cells has been successfully shown (Skordis et al., 2003). Another study reported important parameters to consider for bifunctional oligonucleotides such as chemistry, length of the tail, binding site and the annealing strength to the target site (Owen et al., 2011). Moreover, a recent study showed the in vivo proof-of-concept by injecting the bifunctional oligonucleotides to the brains of the SMA mouse model via intracerebroventricular (ICV) injections. The authors designed two bifunctional oligonucleotides, one that targets a repressor in intron 6 and recruits SR proteins and another one that sterically blocks a silencer in intron 7 and recruits SR proteins. Both of the bifunctional oligonucleotides resulted in protein restoration and enhanced survival (Osman et al., 2012).

Another approach is to use snRNAs in order to manipulate splicing defects. One example is U1 snRNA, which has the ability to sequence specifically bind to the 5'ss and initiate spliceosome assembly **(Figure 9B)**. This has been reported to be efficient with modified U1 snRNAs targeted to the mutated 5'ss in cystic fibrosis (Fernandez Alanis et al., 2012). Moreover, modified U1 snRNAs have also shown to be effective in vivo (Balestra et al., 2014; Denti et al., 2006). In the first report, U1 snRNA expressed from a viral vector has been used to skip mutated exon 23 in mdx mouse model and hence restore dystrophin expression. In the latter report, modified U1 snRNA delivered by adeno-associated viral vector binds to the mutated 5'ss and rescues exon 7 inclusion in the coagulation factor *F7* gene. Moreover, U7 snRNA has also been used for directing splicing. U7 snRNA is natively used for the maturation of histone pre-mRNA. Modified U7 snRNAs with a binding tail for splicing repressor hnRNP A1/A2 has been successfully used for exon skipping, thereby restoring the reading frame in a transgenic mouse model for DMD (Goyenvalle and Davies, 2011). A similar approach has been applied for SMA and modified U7 snRNAs have been shown to enhance exon 7 inclusion in patient-derived SMA fibroblasts (Geib and Hertel, 2009).

A) Mutations create weak 5'ss



B) Modified snRNA



C) Trans splicing

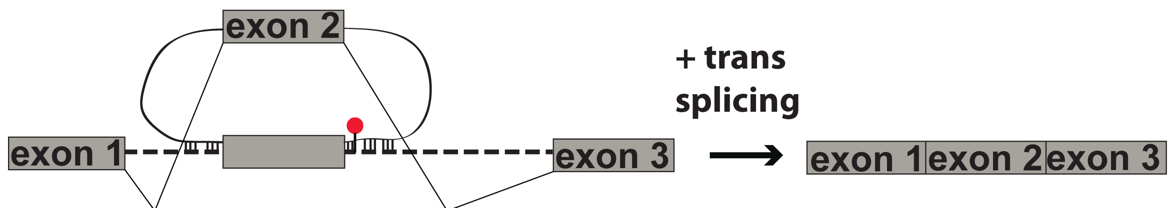


Figure 9. Alternative ways of restoring aberrant splicing. A. Mutations can weaken 5' ss and result in exon exclusion. **B.** Modified snRNAs can bind to the mutated 5' ss and recruit other splicing factors for exon recognition. These snRNAs are usually expressed from vectors. **C.** Trans-splicing RNA with the correct splice sites replaces the faulty exon and the splicing machinery recognizes it as the wild-type exon.

Finally, trans-splicing is also another alternative approach to manipulate splicing (Hammond and Wood, 2011). In this approach, the mutated part of the pre-mRNA can be delivered as pre-trans-splicing molecule (PTM). This approach is mostly used when the GU-AG dinucleotide of the core splice sites are mutated, since these are harder to correct. In this case the delivered PTM includes all the necessary information for splicing and an antisense part for target binding **(Figure 9C)**. This approach has been successfully used in a mouse model to correct a mutation in coagulation factor VIII (Chao et al., 2003).

2 AIMS

The main aim of this PhD thesis is to design antisense oligonucleotides for splicing therapies with a focus on the following areas:

I. Develop a synthesis method for oligonucletide conjugation with the aim of multi-targetting
II. Develop splice-correcting oligonucleotides (SCOs) for the treatment of XLA with the special focus on delivering SCOs to B cells both *ex vivo* and *in vivo*
III. Further develop bifunctional oligonucleotides that can rescue the splicing mutations within the core splice elements in XLA

3 MATERIALS AND METHODS

This section covers some relevant methods used in this thesis, more details and the other methods can be found in the original papers.

3.1 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a semi-quantitative technique and it was applied in all three papers in order to quantify the corrected band in relation to the uncorrected band. For this, sets of primers were designed flanking the regions affected by oligonucleotide treatment. The bands were identified by their distinct size on agarose gel. The analysis and the quantification of the bands were done with QuantityOne Software (Bio-Rad). In paper I, nested PCR was also performed for both *Dmd* and *Mstn*. Details about the conditions and the primers can be found in the original papers.

3.2 Cell culture and transfections

In paper I, mouse H2k mdx muscle cells were grown on gelatinized plates at 33°C, 10% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with glutamax supplemented with 20% fetal bovine serum (FBS), 0.5% chick embryo extract, 200 U/mL penicillin and 200 µg/mL of streptomycin (Invitrogen). H2k mdx myotubes were generated in gelatin coated 24-well plates by seeding 30,000 cells per well, leaving them for 2 days to reach 90% confluency before changing media to starvation media (DMEM with 5% horse serum) and transferring them to 37°C, 5% CO_2 incubator for another 4 days. Cells were transfected using Lipofectamine 2000 (LF2000; Invitrogen) according to manufacturer's protocol and 2.2 µL of LF2000 was used per µg of AON. Complexes were formed in 50 µL OptiMEM (Invitrogen) and added to cells grown in 450 µL full growth media. Cells were analyzed 48 hrs later in all transfection experiments.

In paper II, U2OS/EGFPLucBTKint4mut reporter cells were cultured in DMEM with 10% FBS and maintained at 37°C and 5% CO_2 atmosphere. For AON transfections, cells were plated the day before at a density of 50,000 cells per well in 24-well plate. Transfections were performed using LF2000 according to the manufacturer's protocol and 2.5 µL of LF2000 was used per µg of AON. Complexes were formed in 100 µL OptiMEM (Invitrogen) and added to cells grown in 400 µL full growth media. Cells were further incubated for 24 hrs.

In paper III, U2OS cells were seeded in 24 well-plates at a density of 60,000 cells per well, the day before and transfected using LF2000 the following day. For the transient transfections in U2OS cells, the plasmids and the oligonucleotides were transfected one day after each other (1.8 µL of LF2000 per µg of AON/plasmid). For the B cell lymphoma A20 cells, the transfections were performed by electroporation using Neon Electroporator (Invitrogen) and the 100 µl Tip Kit (Neon Transfection System, Life Technologies). The following settings were used: 1500 V (pulse voltage); 20 ms (pulse width); 2 (pulse number). 800,000 cells were used per well. A20 cells were kept in Roswell Park Memorial

Institute Medium (RPMI;Life Technologies) whereas U2OS were kept in DMEM, both with 10% FBS. Cell were harvested 24 hr after the last transfection.

In paper II, for the naked-uptake strategy in U2OS/EGFPLucBTKint4mut reporter cells; cells were plated the day before to reach 70 % confluency at the day of transfection. After the addition of AONs to the medium, cells were incubated further for 3 days in DMEM containing 10 % FBS. Cells were supplemented with 150 µL medium at day 2.

3.3 DTT cleavage assay and analysis

In paper I, this assay was used to cleave the oligonucleotides linked by a disulfide linker. Freeze-dried antisense oligonucleotides were resuspended in 100mM of 1,4-dithiothreitol (DTT) solution in sodium phosphate buffer (pH 8.3–8.5). The tubes were kept in 37°C overnight as longer incubation time gave a better effect. The samples were run on 15% Tris/Borate/EDTA (TBE) urea gel (Life Technologies) in order to resolve the single-stranded oligonucleotides. Pre-run of the gel was performed for 30 min at 200 V. Samples were mixed with TBE Urea Sample Buffer (2X) (Life Technologies) and the gel was run according to the protocol of the manufacturer. After the run, the gel was stained for 10 minutes with SYBR Gold Nucleic Acid Gel Stain (Invitrogen).

3.4 Flow Cytometry

In paper I, flow cytometry was used to detect the percentage of Cy5-labelled oligonucleotides inside the cells. Before the analysis, the cells were washed extensively with DPBS (Dulbecco's phosphate-buffered saline, Invitrogen) in order to wash away the membrane bound oligonucleotides. Prior to the run, the cells were resuspended in DPBS containing 1 µl /mL propidium iodide (Life Technologies) in order to gate away the dead cells. The cells were analyzed by FACSCalibur instrument (BD) at the Center for Hematology and Regeneration Medicine, Karolinska Institutet. FlowJo software was used to perform the analysis of the data.

In paper II, flow cytometry was used mainly to detect BTK and other B cell markers (see the original paper). The staining protocol for BTK was as follows: B-lymphocytes were incubated in DPBS with CD16/32 (clone 93, Biolegend) and without washing incubated for 30 minutes at 4°C with CD19-PE (clone 1D3, BD). After washing twice with DPBS, cells were resuspended in 1µl/ml DPBS containing LIVE/DEAD Fixable Green Dead Cell Stain (Life Technologies) and incubated for 30 minutes at 4°C. For intracellular staining, the cells were fixed using BD Cytofix/Cytoperm Kit according to manufacturer's protocol. Subsequently, the cells were stained with BTK-Alexa Fluor 647 (clone 53/BTK, BD). The cells were analyzed by FACSCalibur instrument (BD) at the Center for Hematology and Regeneration Medicine, Karolinska Institutet. FlowJo software was used to perform the analysis of the data.

3.5 Splice-correction assay

In paper II, the initial optimization of oligonucleotides was performed in a reporter cell line (U2OS/EGFPLucBTKint4mut). This cell line was constructed from a reporter plasmid in which the luciferase cDNA was interrupted by the introduction of a mutated *BTK* intron 4, containing the studied mutation. Without the oligonucleotide treatment (see section 3.2), the reporter cell synthesizes a corrupt EGFP-luciferase mRNA. However, with an efficient oligonucleotide therapy, the splicing can be rescued and the EGFP-luciferase fusion protein expression is restored. The analysis can be easily made by measuring the luciferase activity. This setup was very helpful to make an initial screening for the efficiency of the oligonucleotides. A similar approach was also used in paper III.

3.6 Primary cell culturing

In paper II, primary B cells were cultured in order to study the splice-correction activity. Primary B cells were purified from the spleen of the mouse using EasySep Mouse B Cell Enrichment Kit, which enriches B cells through negative selection (StemCell Technologies). The purity of isolated B cells was checked by CD19-PE (clone 1D3, BD) and analyzed by flow cytometry. Purity levels were always ≥90%. After isolation, B cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Life Technologies) containing 15% FBS and 50 µM β-mercaptoethanol (Gibco, Life Technologies). For improving the survival of B cells 5 µg/ml CpG ON (obtained from DNA Technology A/S) was added to the cell medium. B cells were always incubated for 3-4 before transfection. CpG was also added 4 hrs after transfection. Primary monocytes were also cultured in the same medium without the addition of CpG.

3.7 Pro-B cell culture

In paper II, pro-B cells were expanded ex vivo for transfection. For the pro-B cell expansion, c-KIT+ cells were enriched from the bone marrow, using immunomagnetic beads (10 µl) (Miltenyi Biotec). After the enrichment the cells were resuspended in OPTIMEM (Life Technologies) containing 10% FBS, 50 µM β-mercaptoethanol, and 100X Penicillin-Streptomycin (Life Technologies). For pro-B cell differentiation the ligands: 10 ng/ml Flk-2/ Flt3 ligand, 10 ng/ml IL-7, and 10 ng/ml KIT ligand (all from Peprotech) were added to the cell suspension. Following this, cells were put on top of OP9 seeder cells that were plated one day earlier. Cells were checked everyday and at day 9, pro-B cells were ready for transfection with 90% purity. Ligands were replenished at day 5.

3.8 Electroporation of primary cells

In paper II, transfection of primary cells was performed by electroporation using a Neon Electroporator (Invitrogen) and the 100 µl Tip Kit (Neon Transfection System, Life Technologies). Primary B cells required high voltage for oligonucleotide uptake. The following settings were used: 2,100 V (pulse voltage); 20 ms (pulse width); 1 (pulse number). The settings for monocytes were as follows: 1,800 V (pulse voltage); 30 ms (pulse width); 1

(pulse number). T buffer was used during the electroporation as required by the manufacturer. It was very important not to leave the cells for more than 5-10 minutes in the T buffer, in order not to damage the cells. Maximum oligonucleotide volume was never more than 10% of the total resuspension volume of the Neon Tip. After electroporation, the cells were transferred to a 24-well, non-tissue culture-treated plate (Falcon, BD). Monocytes were incubated for 4 hrs, after which an additional volume of 500 µl medium was added. B cells were incubated for 4 hrs, after which an additional volume of 500 µl medium was added together with 5 µg/ml CpG ON.

For the gymnosis/naked uptake experiments, cells were plated at $1.5x10^6$ cells per well after 4 hrs of incubation with CpG. B-PMOs were incubated at 37^oC for 30 min and sonicated for few seconds before adding to the cells. The cells were incubated further 3-4 days.

3.9 Western Blot and Immunoprecipitation

In paper II, immunoprecipitation was performed using Dynabeads protein G (Life Technologies) according to the manufacturer's protocol. In paper II and III, western blot was performed as follows; cells were lysed with lysis buffer (50 mM HEPES, pH 7.0, 120 mM NaCl, 10 % glycerol, 1% NP-40, 0.5% sodium deoxycholate), supplemented with protease inhibitors (Complete Mini, Roche) for 30 minutes with vortexing. Finally, the lysates were centrifuged and supernatant was taken. Proteins were separated on gradient 4%–12% SDS Bis-Tris NuPAGE gels (Life Technologies) and transferred onto nitrocellulose membranes using the Iblot system (Invitrogen). The membranes were then blocked with LI-COR Blocking Buffer (LI-COR Biosciences GmbH) and probed with specific primary antibodies.

In paper II, the following primary antibodies were used for detection: actin (A5441, Sigma-Aldrich), BTK (270-284, Sigma-Aldrich), phospho-BTK (pY551) (558129, BD), and BTK (611117, BD). The following secondary antibodies were used for detection: goat anti-mouse 800 CW, goat anti-rabbit 800 CW, goat anti-mouse 680 LT, or goat anti-rabbit 680, all from LI-COR Biosciences GmbH.

In paper III, the following primary antibodies were used for detection: Anti-TDP-43 (ab41881, Abcam), anti-TIA-1 (sc-1751, Santa Cruz), anti-actin (A5441, Sigma-Aldrich). The following secondary antibodies were used for detection: anti-goat 800CW, anti-rabbit 680LT, all from LI-COR Biosciences GmbH.

Western blots signals were scanned by using Odyssey Imager from LI-COR Biosciences GmbH. Quantitative analysis of proteins were calculated as the percentage of relative intensity by ImageJ software according to the manufacturer's protocol.

3.10 Anti-IgM stimulation and cell survival assay

In paper II, in order to check for the phosphorylation state of BTK, 48 hrs after electroporation B cells were starved for 6 hrs in IMDM, containing only 50 µM β-mercaptoethanol. Following this, cells were pelleted and resuspended in $5x10^6$ cell per ml in DPBS. Then anti-IgM was added to a concentration of 20 µg/ml, and cells were incubated for exactly 1-minute and harvested for analysis.

In paper II, in order to check for the viability of the cells, 48 hrs after electroporation, B-lymphocytes were washed to remove the CpG and resuspended in complete medium with $2x10^6$/well. Following this 20 µg/ml anti-IgM was added to each well and cells were incubated further for 24-48 hrs. Cells were counted with Bürker chamber and trypan blue exclusion method was used to evaluate the viability of the cells. Anti Igm: Goat $F(ab')_2$ anti-mouse IgM (1022-01, Southern-Biotech).

3.11 Injection to the BAC-transgenic mice

In paper II, the B-PMO was injected to the BAC-transgenic mice. B-PMO was prepared in 200 µl saline with the required concentration and injected into the tail vein. The mice received 30 mg/kg B-PMO or only saline every second day for 3 injections in total. After the last intravenous injection, the mice were injected subcutaneously with another 30 mg/kg B-PMO or only saline to increase the time of circulating AON. The mice were sacrificed 7-8 days after the first tail vein injection.

4 RESULTS AND DISCUSSION

4.1 Paper I

Solid-phase synthesis of bispecific oligonucleotides with a disulfide linker can be used for multitargeting strategy.

In this paper, we have developed a solid-phase synthesis method in order to conjugate several antisense oligonucleotides (AONs) via cleavable disulfide linker. In contrast to the cocktail approach where mixed AONs were used for multitargeting, here we aimed to achieve equimolar delivery by using bispecific oligonucleotides, rendering two functional moieties linked by a cleavable linker. These bispecific oligonucleotides are cleaved inside the reductive environment within the cytoplasm of the cell, releasing two separate antisense moities.

For some diseases it is desirable to simultaneously target several transcripts or even different positions within the same transcript in order to have a robust therapeutic effect (Aartsma-Rus et al., 2006). Moreover, a dual targeting approach has already been shown with SSOs linked by a uracil containing spacer linker, however, using such linker the effects were relatively low (Aartsma-Rus et al., 2004). To this end, we sought to target myostatin (*Mstn*) and dystrophin (*Dmd*) for the treatment of Duchenne Muscular Dystrophy (DMD). Myostatin has been reported to inhibit muscle growth and lack of dystrophin is the cause of DMD (explained in section 1.2.1.4). Thus, using the bispecific approach, we aimed to inhibit *Mstn* (via exon 2 skipping) and enhance production of in-frame *Dmd* (via exon 23 skipping) at the same time. We tested our concept in mouse immortalized H2k muscle cells as well as in mdx mouse model.

We initiated our study by finding an antisense oligonucleotide (AON) sequence that would skip exon 2 in *Mstn*, introducing a premature termination codon (PTC). We based our optimization on a previous study, in which the authors targeted exonic enhancer regions of exon 2 (Kang et al., 2011). After cell culture screening, our analysis showed that AON3 was the most efficient in exon 2 skipping. It was in a similar range with the previously published sequence, but nevertheless we decided to move on with our optimized AON. For exon 23 skipping in *Dmd*, we decided to use a previously published AON (Ex23D (+7-18)), which is targeting the exon-intron junction at 5'ss of exon 23 (Mann et al., 2002). To combine the two functionalities, we designed and synthesized bispecific SSOs that are linked by a cleavable disulfide linker (BI-S) or by a non-cleavable carbon-18 linker (BI-C18). Analysis of exon 23 skipping in cell culture experiments revealed that BI-S SSOs are as good as the monospecifics, i.e., non-conjugated versions in promoting exon skipping. As we expected, BI-C18 SSOs with a non-cleavable linker showed the least activity among all. Moreover, a double skipped product upon SSOs treatment was also observed by RT-PCR, prominently increasing in exon 23 skipped samples. This product is an exon 22-23 skipped transcript that has been previously reported by others (Mann et al., 2002; Wilton et al., 1999). It might be that SSOs targeting the exon-intron junction of exon 23 might disrupt the “exon definition”

which also involves the 3' splice-site (3'ss) of intron 22. This in turn could reduce the recognition of exon 22 by the spliceosome and favor exons 21 and 24 to be joined. In addition, the disruption of exon 22-23 recognition might make it easier for the exons 21-24 to compete for the available splicing factors. Interestingly, double skipped products are greatly reduced or absent in primary cell culture or in vivo experiments, clarifying the differential cellular environment affecting splicing. In addition, analysis of myostatin skipping also showed reduced effect in BI-C18 compared to monospecifics and BI-S. However, it should be emphasized that the effects were not as significantly different as they were in exon 23 skipping and even at low concentrations we could detect efficient exon 2 skipping. Nevertheless, our cell culture experiments prove that conjugated SSOs were functional and were able to target two different transcripts. We then decided to test the efficiency of BI-S SSOs in vivo, in the mdx mouse model. Unfortunately, we were unable to detect any *Dmd* transcript but only exon 2 skipped *Mstn* transcripts. The reason might be attributed to the shorter transcript length of *Mstn* (3 exons) compared to *Dmd* (79 exons), potentially resulting in higher transcription rate and thus providing more available *Mstn* targets for the SSOs. On the other hand, longer transcription time for *Dmd* might also give more time for the SSOs to find their target but still the overall turnover would be less compared to *Mstn*. However, *Mstn* turnover being higher is just a speculation and needs to be investigated. The reason might just turn out to be that exon 2 skipping is more efficient due to a shorter pre-mRNA length of *Mstn*. On other hand, poor delivery of bispecific SSOs in vivo combined with the less efficient activity of *Dmd* targeting arm, might in part explain the absence of exon 23 skipping in vivo. The lower efficiency of exon 23 skipping in mdx mouse model by the same sequence has been reported before (Heemskerk et al., 2009).

Following this, we also tested if the BI-S SSOs had transfection advantage over the monospecific versions. Since BI-S SSOs have more charge, Lipofectamine 2000, which is a cationic lipid, might have better complexation efficiency compared to the monospecific SSOs. We transfected Cy-5 labeled BI-S as well as the monospecific SSOs and performed FACS analysis to quantify the signal. Quantification analysis revealed that BI-S SSOs did not have a transfection advantage over the monospecific versions. Moreover, we also tested if BI-S would cause increased toxicity in the cells as long stretches of PS-modified AONs might have unspecific protein interactions in the cell. However, even at high concentrations (200nM), we did not observe any toxicity in the cell culture.

We also tested if the orientation of SSOs would matter for their efficiency. For this reason we synthesized invBI-S, which had the *Mstn* targeting AON at the 5'-end. The analysis revealed that the internal orientation did not make any difference for the activity.

Following repeated experiments with several freeze/thaw cycles, we realized that BI-S SSO lost its activity, although monospecific versions were relatively stable. To check the integrity of the BI-S SSOs, we used the reducing agent DTT and analyzed the cleavage efficiency on a polyacrylamide gel. As expected, after DTT treatment BI-S released the two oligonucleotides and BI-C18 remained intact. However, BI-S compounds that were exposed to several freeze-

thaw cycles turned out to be cleaved even without the DTT treatment. To address this stability issue we tested another internal disulfide linker (DTPA) that is supposed to have slower cleavage kinetics and therefore be more stable. For this reason, we synthesized new bispecific SSOs, BI-XS, with the DTPA linker and this time we used a shorter version of *Dmd* targeting oligonucleotide, owing to easier synthesis on a larger scale. Once cleaved in the intracellular environment, BI-XS SSO releases the monospecific compounds without the 5' and 3'-thiol groups in contrast to the BI-S SSO, i.e., more similar to the monospecific versions. To this end, one can speculate that the reduced effect of BI-S SSOs might be due to the free thiol groups exposed upon degradation and affecting transfection or complexation efficiency of the SSOs. However this is pure speculation and needs to be confirmed. Moreover, upon DTT treatment, BI-XS also released the separate monospecific SSOs albeit with reduced efficiency as expected. We further on tested the efficiency of BI-XS in cell culture experiments and although both *Dmd* exon 23 skipping and *Mstn* exon 2 skipping were marginally reduced, they remained effective.

In conclusion, we have optimized a solid-phase synthesis method for conjugation of two functional moieties with a cleavable disulfide linker and the method can be applied using commercially available reagents. Our proof-of-concept study shows that bispecific SSOs are as efficient as their monospecific versions and better than the non-cleavable ones. In comparison to the previous studies where a similar approach has been applied for siRNAs (Chung et al., 2011), our compounds are homogenous and can be obtained in high yields with a solid-phase synthesis method. Bispecific approach can be applied for targeting two different places in the same transcript and cooperative effect of SSOs might result in a better effect as shown by the DMD studies. One can also apply similar strategy with different chemistries as recently shown by using a CPP-PMO based system (Shabanpoor et al., 2015). Despite the exciting possibilities of the bispecific approach, delivery of this relatively large molecule is still a hurdle and needs to be optimized.

4.2 Paper II

Splice-correcting oligonucleotides rescue an intronic mutation in XLA and restore the functional BTK both ex vivo and in vivo.

In this paper, we have studied the possibility of correcting an intronic mutation in a primary immunodeficiency disease model, X-linked agammaglobulinemia (XLA). As discussed in section *1.2.1*, XLA is caused by the lack of Bruton’s Tyrosine Kinase (BTK), a crucial protein for B cell development. XLA is caused by different kinds of mutations and in this paper we studied a specific one in the intron 4 of *BTK*, which was previously identified in our laboratory. The mutation is an A to T change in the intron 4 of *BTK* that activates an already existing cryptic splice-site, resulting in an inclusion of 109 bp of a pseudoexon between exons 4 and 5. This changes the reading-frame and creates a PTC, abolishing the translation of BTK possibly with non-sense mediated decay (NMD).

This prompted us to design and use splice-correcting oligonucleotides (SCOs) for rescuing BTK expression. We first started by designing a reporter construct where we inserted the patient-derived intron 4 into the Firefly Luciferase expression cassette fused with EGFP. We then prepared a reporter cell line in order to be able to screen for highly active SCOs, using the human osteosarcoma U2OS cell line. The underlying rationale of this reporter cell line is that if the mutation is successfully blocked, the correct splicing results in EGFP-luciferase mRNA, which can easily be detected by checking the luminescence activity. We designed a battery of 2'-OME-PS-modified SCOs that target the 5'ss and 3'ss as well as the several exonic enhancer regions of the pseudoexon. Several bioinformatics tools that have been published before were used to identify the enhancer regions (Cartegni et al., 2003; Fairbrother et al., 2002; Zhang and Chasin, 2004). The initial screening of the 2'-OME-PS-modified SCOs revealed that targeting the 5'ss of the pseudoexon region was better in efficiency compared to targeting the 3'ss. This might be because the 3'ss region might contain secondary structures, making it harder for the SCOs to bind. Alternatively, the SCOs might change the secondary structure and improve the binding of an enhancer protein to the region. Therefore, we chose to continue to the next phase with SCO 187.18, targeting the 5'ss of the pseudoexon. In addition, we chose 186.18, targeting the central region of the pseudoexon and 190.18 targeting just downstream of the 3'ss of pseudoexon. Although they were less efficient compared to 187.18, we wanted to test if changing the chemical modifications would affect their activity.

In the next phase, we further modified the candidate SCOs by shortening their length (18nt to 15 nt) and inserting some LNA nucleotides (LNA-2'-OME-PS mixmers) in order to increase their target affinity. Shorter SCOs are known to have an advantage for easier delivery as well as cellular uptake in vivo (Gupta et al., 2010; Straarup et al., 2010). Moreover, proper design of shorter SCOs with increased affinity to its target is also reducing the risk of off-target effects (Elayadi et al., 2002). Reporter cell line analysis revealed that LNA modifications of 186 and 187 improved the efficiency of SCOs, especially at lower concentrations. We decided to drop the SCO targeting the 3'ss of pseudoexon, since its LNA-modified version (190.15) was toxic for the cells at high concentrations, probably due to off-target effects.

Due to their efficiency, we further wanted to modify the 186 and 187 series, making them even shorter as well as exploiting other possible chemical modifications. To this end, we also reduced the LNA content of the 186.15 sequence and designed 186.15red with only three LNA substitutions. This new design turned out to be very efficient, even better than the 187.15 SCO, and owing to its low LNA content, a less risky option for off-target effects. Moreover, shorter versions of 187 and 186 series (10-12 mers) did not show any advantage in efficiency. This may be attributed to a possible threshold in length, due to the context of teh target region, as certain places in the pre-mRNA might form secondary structures and SCOs with a certain length might be needed for effective binding. On the other hand, several other modifications such as amino-glycyl-LNA, unlocked nucleic acid (UNA) and ZEN modifications did not show any advantage over the solely LNA modified 2'-OME-PS SCOs. Amino-glycyl-LNA with its positive charge would increase target efficiency due to decreased

charge repulsion (Johannsen et al., 2011) and UNA with its decreased binding efficiency would decrease off-target effects (Campbell and Wengel, 2011). Moreover, a ZEN modification (a non-base modifier) is known to increase the efficiency of 2'-OME oligonucleotides (Lennox et al., 2013). To study whether these had any effect, we also designed two more SCOs targeting the GAA-triplet regions, around the 5'ss of the pseudoexon. The underlying reason for targeting the GAA-triplet regions was that they are known to be present in certain enhancer regions (Kralovicova et al., 2011; Yeakley et al., 1996). However, we did not observe any improvements. Taken together, these results show that none of these SCOs were better than the 186.15red LNA modified 2´-OME PS SCO. I should also add here that we did not systematically analyze and compare these modifications further, since our ultimate aim was to find a good candidate SCO for in the vivo experiments.

The initial screenings were done with the aid of a lipid-based transfection reagent. We also wanted to test if the cells can take up our candidate SCO without any transfection reagent. This concept is called "gymnosis" and it has been proposed to have a better correlation with the in vivo efficiency (Stein et al., 2010). The underlying mechanism is not very well defined, yet the approach can be used to predict the internalization capacity of the SCOs. However, it is not straightforward to correlate the in vitro and in vivo results since they can have different mechanisms. For the gymnosis strategy, we also designed a PMO-modified version (186.25) of the 186 SCO as well as a cell-penetrating peptide (CPP) conjugated version thereof, B-PMO 186.25. We chose the arginine rich B-peptide, since it has been shown to be efficient in DMD studies with even delivery to the heart (Yin et al., 2008) and the brain in an another setting (Du et al., 2011). Gymnosis experiments in the reporter cell line resulted in high potency of the LNA-modified 186.15red and the B-PMO.

After the reporter cell line experiments, it was time for us to see the effect of our candidate SCOs in a more relevant setting using a mouse model. We generated a BAC (Bacterial Artificial Chromosome) transgenic mouse model, harboring the full-human *BTK* locus with the intron 4 mutation. These mice were bred onto *Btk* KO mice in order to generate a transgenic mouse model that is only expressing mutated human *BTK*, namely BAC-transgenic mice. I should also note here that our mouse model has a similar phenotype with the *Btk* KO and the xid mice, harboring 50% of immature type B cells in the periphery, whereas in XLA patients this is hardly 1%.

We tested the efficiency of the SCOs in an ex vivo treatment in B cells isolated from the spleen of the BAC-transgenic mice. Splenic B cells turned out to be hard to transfect, and we were able to deliver the SCOs only by using electroporation. In the other transfection experiments, one can speculate that the SCOs may be able to get in to the cells but are then entrapped either within the vesicles or endosomes. Since B cells are immune cells, they might have higher thresholds for internalization. Nevertheless, 48 hours after electroporation with 186.15red SCO, we were able to observe restored BTK both at the protein and RNA level. The FACS analysis showed over 35% of the B cells with a positive BTK signal.

Subsequently, we wanted to test if the restored protein is functional after splice-correction ex vivo. To do this, we tested the survival of B cells upon activation through the B cell receptor after the restoration of BTK (48 hours after electroporation). It is known that anti-IgM stimulation through the B cell receptor induces B cell proliferation in the presence of BTK (Khan et al., 1995). Our analysis indicated that SCO-treated B cells had proliferative advantage whereas the non-treated cells died completely. In addition, we substantiated our hypothesis with another functional test by checking the phosphorylation state of the restored protein. For BTK to be catalytically active it has to be phosphorylated at tyrosine Y551 upon IgM stimulation and this enables its involvement in the downstream pathways (Mohamed et al., 2009). In a similar approach upon IgM stimulation of the SCO treated B cells, we could detect very significant levels of tyrosine Y551 phosphorylation. These functional tests thus verified that the efficiency of BTK restoration was sufficient to make it functionally active in the B cells.

Once again, we also tested the naked uptake efficiency of three SCOs, 186.15red, PMO 186.25 and B-PMO 186.25. Our analysis revealed that only B-PMO 186.25 was efficient in restoring BTK, and at very low levels. This result again reveals the poor correlation of in vitro and in vivo results. Moreover, owing to the fact that B cell are suspension cells, it might be much more difficult for the SCOs to target the cells for internalization compared to the adherent U2OS reporter cell line. In this instance, efficient CPP internalization capability through the cell membrane might be the crucial component. In addition, different SCO chemistries might have different kinetics for internalization, such that longer incubation times and different dosing regimen might be needed for 186.15red and PMO 186.25.

As a follow-up, we then moved on to test the possibility of targeting progenitor B cells. XLA is a B cell development disorder, and the targeting priority is not the for the splenic B cells but rather for the pro-B cells that reside in the bone marrow. For this reason, we expanded stem cells taken from the bone marrow and differentiated them to pro-B cell ex vivo. We then applied the electroporation with the SCO 186.15red, and after 48 hours restoration of BTK was observed. This was one of the key points for our study, since we could show that our strategy works also for the real target cells. To this end, we decided to administer the B-PMO conjugates systemically to the BAC-transgenic mouse model. After a week of treatment, we sacrificed the mice for protein and RNA analysis. This revealed the restoration of BTK both in the spleen and in the bone marrow of the treated mice. More importantly, we isolated the B cells from these two organs and we could show the restoration of BTK. This was very exciting since, not only could the B-PMO conjugates restore BTK expression, but they also could locate into the bone marrow and be internalized by the B cells.

As a final proof-of-concept we applied our strategy to primary patient cells. We took monocytes from the XLA patient, as the patient does not have B cells but only monocytes with the mutated *BTK*. We applied electroporation as well as the naked uptake strategy and restoration of BTK was observed in both settings. An important note here is that, although lower amounts of SCOs were applied in monocytes compared to B cells, the restoration of

BTK was much more drastic in monocytes. This can be attributed to the tougher internalization requirements of the B cells as well as the lower survival rate during the culturing, since transfected cells might die during the incubation lowering the overall efficiency of transfection.

In conclusion, for the first time we could show the possibility of splice-correction in primary B cells both ex vivo and in vivo. Moreover, it was also novel to show that B-PMO could actually target the B cells in the bone marrow. As a future therapy option, one can apply ex vivo treatment of the pro-B cells and inject them back to the patient or, alternatively, SCOs can also be injected systemically (Bestas et al., 2015). To this end, the precise administration route might be very crucial for an efficient restoration. One might inject the SCOs directly to the bone marrow where differentiation of the B cells occurs. At this point, there is also another aspect to consider; for the rescued B cells to become mature plasma cells they need to be exposed to antigens inside the lymph nodes. One might speculate here that a supplementary therapy might be to inject the antigens together with SCOs directly into the lymph nodes in order to make sure that the circulating, SCO-rescued B cells are exposed to antigens. After antigen exposure, they would then become mature plasma cells, which do not need BTK anymore. All in all, our proof-of-concept study opens up many avenues for the targeting of hematopoietic cells.

4.3 Paper III

Bifunctional splice correcting oligonucleotides (bifSCOs) can potentially be used to rescue core splicing mutations in XLA.

In this study, we explored the possibility of using bifunctional splice-correcting oligonucleotides (bifSCO) in order to rescue core splice site mutations identified in XLA patients. The bifSCOs have an antisense region binding to the target pre-mRNA as well as a free tail of modified RNA for recruiting splicing factors to the site. This strategy has been successfully used especially for SMA in several studies (Brosseau et al., 2014; Osman et al., 2012; Owen et al., 2011; Skordis et al., 2003).

We initially gathered six different mutations that are located near the core splice sites flanking either exon 16 or exon 17. We then prepared seven different reporter constructs each containing *BTK* cDNA, and also including the introns separating exons 15-18 with the intervening introns. Six reporters contained one of the patient mutations each. In addition, *BTK* cDNA was designed to be fused with Firefly Luciferase cDNA for easier screening of the bifSCOs.

We initiated our study by first checking the splicing patterns of the reporter constructs. Transient transfection of the constructs into human osteosarcoma U2OS cell lines identified complex splicing patterns. The construct that harbors a deletion at the polypyrimidine tract (PPT) of intron 15 (i15ΔPPT) mainly resulted in exon 16 skipping, as expected, since deletion at the PPT site disrupts U2AF binding. In addition, we observed another, higher molecular weight product which turned out to be caused by the activation of an upstream

cryptic 3'ss in intron 15. Most probably, disruption of the PPT weakens the 3'ss of intron 15 and the spliceosome favors an upstream cryptic site. Constructs that are harboring mutations at the 5'ss of intron 16 (i16T+2C and i16G+5T) also resulted in higher molecular weight products. These products turned out to be caused by the activation of an alternative, cryptic 5'ss which is 60 nt downstream of the true 5'ss. Moreover, sequencing of the other, smaller products revealed a mixture of two isoforms. One of the isoforms was the exon 16 skipped product, as expected. The other isoform, however, was formed by exon 17 skipping combined with inclusion of the lengthened exon 16 spliced at the cryptic 5'ss. One can speculate that exon 17 splicing depends on exon 16 and/or intron 16 definition. In support of this hypothesis, exon 16-17 skipping was observed in all mutant constructs, although at low levels. On the other hand, the intrinsic weak splice-sites of exon 17 might also be the reason, since even in the wild type construct one can observe exon 17 skipping. Moreover, when we analyzed the reporter constructs harboring mutations around exon 17, the scenario turned out to be much more straightforward. All the mutations flanking exon 17 resulted mainly in exon 17 skipping, whether in upstream intron 16 3'ss (i16C-3A), in exon 17 by the 5'ss (e17G-1A) or in intron 17 (i17G+5A).

We then decided to design bifSCOs for rescuing these defects despite their complexity. We reasoned that it would be beneficial to target the bifSCOs to the introns in order to block the activated cryptic splice sites. We then decided upon two different tails intended to recruit two different splicing factors, TDP-43 and TIA-1. The reasoning for TDP-43 was that it binds to UG-rich intronic regions and enhances the binding of U1snRNP to 5'ss, and this particular tail has been shown to function in a bifSCO in a recent publication (Brosseau et al., 2014). Moreover, TIA-1 has also been shown to bind U-rich intronic sequences by cross-linking/immunoprecipitation studies, and likewise shown to recruit U1snRNP to 5'ss (Forch et al., 2002; Wang et al., 2010). Accordingly, we designed bifSCOs that are targeting intron 16 (45nt downstream of the 5'ss) and intron 17 (27nt downstream of the 5'ss) and conjugated either to a TDP-43 tail or a TIA-1 tail. In addition we chose to use 2'-OMe PS modification for the antisense part and 2'-OMe modification for the tail part, having only PS in the last three nucleotides in order to increase stability. One should also note here that pure RNA tail with a PS modification in the end has been shown to be more effective compared to 2'-OMe PS modification (Owen et al., 2011; Skordis et al., 2003). For our purpose, we decided to start with a 2'-OMe PS modified tail instead in order to be on the safe side regarding stability/degradation issues.

We analyzed the efficiency of bifSCOs by transient transfection together with the reporter constructs into the U2OS osteosarcoma cell line. The most dramatic effect was observed with the i15ΔPPT mutant, as a corrected product prominently appeared in RT-PCR. No significant difference between the two tails was observed. Intron 16 mutants (i16T+2C and i16G+5T), also showed rescue, albeit at lower levels. However, the enhancement of the exon 16-skipped product was more prominent than the enhancement of the correct product. So, despite blocking the cryptic splicing event, bifSCOs are not optimal for suppressing exon 16 skipping. One might speculate that the bifSCO needs to be closer to the 5'ss to be more

effective, since in this case it is 45nt downstream. However, with the presence of the cryptic splice site this is challenging, and one might need to use a dual approach, i.e., one oligonucleotide blocking the cryptic site and another one to act as the bifSCO in proximity to the 5'ss. Compared to the exon 16 mutants, analysis of the exon 17 mutants revealed much lower rescue by bifSCOs, and only worked for two of them. This might again be attributed to the weaker recognition of this exon. However, our preliminary data presented here indicate that there seems to be a preference for the TDP-43 tail, suggesting that regulation by specific splicing factors may also differ between exons 16 and 17.

We also tested the functionality of bifSCOs in another cell line, HeLa cells. This experiment also revealed the context dependency of splicing factors within different cellular environments. For example, the i15ΔPPT mutant showed much more cryptic splice site activation and much less rescue. Here the preference for TDP-43 for exon 17 mutants was also not prominent anymore. This experiment therefore underscores a very important parameter to consider for the design of the bifSCOs, namely target cells.

After the initial screening of bifSCOs we decided to prepare stable cell lines with the above-mentioned reporters. The primary reason for doing this is that transient transfections are not always homogenous and it is much more relevant to see the effect of bifSCOs on RNA transcribed in a genomic context rather than from a plasmid. In these settings the uncorrected products gave a very weak signal in RT-PCR, yet upon treatment both the correct and the exon-skipped bands were enhanced. As before, upon bifSCO treatment rescue was observed for all mutants, except the i17G+5A. An interesting point here is that the enhancement of the cryptic band (due to the alternative 5'ss in intron 16) in exon 16 flanking mutations was not prominent anymore. This might be attributed to the difference of expression from a genomic locus as this might affect transcription and stability differences might become more obvious compared to transient transfections. Therefore, we decided to check for a possible involvement of NMD. We selected two stable cell lines, i16G+5T and i16C-3A, and treated the cells with translational cycloheximide (CHX) after bifSCO treatment. This experiment revealed an even more complex pattern. Upon CHX treatment untransfected i16G+5T cells showed the most effective stabilization for the cryptic band and surprisingly a minor stabilization was also observed for the wild type band. In contrast, only the exon 17-skipped product stabilization was observed for i16C-3A. For the bifSCO-treated samples in both mutants, CHX treatment did not seem to have an effect on the rescued band, despite the fact that the efficiency of rescue was low. This experiment reveals that different isoforms from both mutants have differing stabilities. However, further experimentation is required to ascertain the precise role of NMD and/or other degratory pathways, and rule out possible experimental artifacts.

As a final proof-of-concept, we performed transient transfection in a more relevant cell type, a B cell lymphoma line A20. Since our ultimate target is a B cell, it is important to check the efficiency of the bifSCOs in a more relevant context. Although the levels were low, we could still detect rescue in both mutants. Similar to the stable cell line experiments, we observed

enhancement of the double-skipped bands upon bifSCO treatment. I should also add here that the overall levels of expression were low in the A20 experiments. This might be due to the reporter plasmids harboring CMV promoter, which might not be optimal for the B cells. Here, reporter i15ΔPPT has even somewhat lower expression level than the other mutants. Interestingly, only a faint rescued band can be observed upon bifSCO treatment, dissimilar to the other exon 16 flanking mutants. This might hint that i15ΔPPT reporter is providing a more straightforward system for bifSCO-mediated rescue.

In conclusion, we were able to show that core splice site mutations can be partially rescued by bifSCOs. Our work is still at its infancy and definitely seems to be challenging due to unexpected cryptic splice sites and regulatory pathways that we have not defined yet. More optimization is needed regarding the binding site of the bifSCO, chemistry and also the protein to recruit. We have not proven as yet if the expected proteins are tethered to the RNA binding tail. Mass spectrometry analysis must be performed in order to detect the proteins binding to the RNA tail and further optimize the bifSCO design. Although challenging, the possibility of rescuing the core splice site mutations is exciting and seems to be applicable if the bifSCOs are carefully designed.

5 CONCLUDING REMARKS

Manipulation of mis-splicing with oligonucleotide therapeutics is quite a challenging approach, and requires taking into consideration several important parameters for each step. For the delivery, it is important to consider the target organ, dosing regimen, biocompatibility and biological half-life of the therapeutic as well as the administration route. On the other hand, in the microscale of the events, the phenotype of the target cell is one of the most crucial parameters. Some cells might be more prone to internalizing the therapeutics much easier, whereas some might need more advanced modifications. To this end, the type of the chemical modification as well as the length of the oligonucleotide are important parameters that also affect the nuclear delivery and, ultimately, the pre-mRNA targeting. The optimal design should take into consideration target specificity, long biological half-life as well as efficient binding to the pre-mRNA.

The results in this thesis address the design of oligonucleotide therapeutics for different mutational settings, as well as targeting multiple transcripts simultaneously. One of the most crucial findings is about the preferred oligonucleotide design for B cell uptake and successful splice manipulation within these cells, including in an in vivo settings. The results suggest that LNA modified therapeutics are very efficient in splice manipulation, yet the delivery issue has to be resolved. In contrast, B cells efficiently take up PMOs conjugated to cell-penetrating peptides (CPPs) and in an in vivo setting they can reach the bone marrow and restore splicing. Perhaps, investigation of different CPP variants might be beneficial to improve the efficiency while lowering the dose. On the other hand, the preliminary findings in another mutational setting underscore the importance of designing optimal therapeutics for the correction of core splice site mutations and highlight its challenges. Mutations in the core splice sites seem to activate other cryptic splicing events and depending on the cell type the activity of the therapeutics might change. Thus, understanding the mis-splicing mechanism to be rescued, the target cell and the optimal modification for cellular uptake are of crucial importance for an efficient oligonucleotide therapy.

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