MOLECULAR AND PRECLINICAL ASPECTS OF ANTISENSE OLIGONUCLEOTIDE TREATMENT FOR MYOTONIC DYSTROPHY TYPE 1

Anchel González Barriga
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Cover image: artistic impression of the (CUG)n repeat that is present in expanded DMPK transcripts of myotonic dystrophy type 1 patients (molecular cause of the disease), forming a characteristic hairpin structure on the back cover and being targeted by an antisense oligonucleotide on the front cover. The antisense oligonucleotide drawing is inspired by the logomark of Prosensa Therapeutics.

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MOLECULAR AND PRECLINICAL ASPECTS OF ANTISENSE OLIGONUCLEOTIDE TREATMENT FOR MYOTONIC DYSTROPHY TYPE 1

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by

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“If I have seen further it is by standing on the shoulders of Giants”

Isaac Newton to Robert Hooke (1676)
ABBREVIATIONS & GENE NOMENCLATURE

2’-F 2’-Fluoro
2’-MOE 2’-O-methoxyethyl
2’-OMe 2’-O-methyl
A Adenine
a.u. Arbitrary units
AAV Adeno-associated virus
ACTA1* Actin, alpha 1, skeletal muscle
ACTB* Actin, beta
AGO2* Argonaute 2, RISC catalytic component
AIDS Acquired immune deficiency syndrome
aka Also known as
AON Antisense oligonucleotide
Ara-C Cytosine β-D-arabinofuranoside
ATP Adenosine triphosphate
BNA Bridged nucleic acid
bp Base pairs
C Cytosine
CDM Congenital myotonic dystrophy type 1
cDNA Copy/complementary deoxyribonucleic acid
CELF1* CUGBP, elav-like family member 1 (aka CUGBP1)
cET Constrained-ethyl
CHQ Chloroquine
CK* Creatine kinase
CLCN1* Chloride voltage-gated channel 1
CNBP* CCHC-type zinc finger nucleic acid binding protein (aka ZNF9)
CNS Central nervous system
Cy3 Cyanine dye family member 3
DDX5* DEAD (Asp-Glu-Ala-Asp) box helicase 5
DGC Dystrophin-glycoprotein complex
DM Dystrophia myotonica
DM1 Myotonic dystrophy type 1
DM2 Myotonic dystrophy type 2
DM500 Mouse model derived from the DM300-328 line with a (CTG)500 repeat
DMD Duchenne muscular dystrophy
DMD* Dystrophin
DMEM Dulbecco’s modified eagles medium
DMPK* Dystrophia myotonica protein kinase
DMSO Dimethylsulfoxide
DMSXL Mouse model derived from the DM300-328 line with a (CTG)>1200 repeat
DNA Deoxyribonucleic acid
dNTP Deoxyribonucleotide
DTT Dithiothreitol
(E)GFP (Enhanced) green fluorescence protein
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBD</td>
<td>Evans blue dye</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF2A*</td>
<td>Eukaryotic translation initiation factor 2A</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENA</td>
<td>2'-O,4'-C-ethylene-bridged nucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESE</td>
<td>Exonic splicing enhancer</td>
</tr>
<tr>
<td>FAM</td>
<td>Carboxyfluorescein</td>
</tr>
<tr>
<td>FANA</td>
<td>Fluoro-arabino nucleic acid</td>
</tr>
<tr>
<td>FEN1*</td>
<td>Flap structure-specific endonuclease 1</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GAPDH*</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GEP</td>
<td>Gene expression profiling</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington's disease</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>HSA*</td>
<td>Human skeletal actin large repeat (DM1 mouse model)</td>
</tr>
<tr>
<td>HTT*</td>
<td>Huntingtonin</td>
</tr>
<tr>
<td>I</td>
<td>Inosine</td>
</tr>
<tr>
<td>i.c.v</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IFN-γ*</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Lamp1*</td>
<td>Lysosome associated membrane protein 1</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
</tr>
<tr>
<td>MBNL*</td>
<td>Muscleblind like splicing regulator 1</td>
</tr>
<tr>
<td>mdx</td>
<td>Mouse model of Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NMD</td>
<td>Nonsense mediated decay</td>
</tr>
<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PKC*</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKR*</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PMO</td>
<td>phosphorodiamidate morpholino oligomer</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PNA</td>
<td>Peptide nucleic acid</td>
</tr>
<tr>
<td>PO</td>
<td>Phosphate</td>
</tr>
<tr>
<td>Poly-Q</td>
<td>Polyglutamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PROMM</td>
<td>Proximal myotonic myopathy (DM2)</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphorothioate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNase H*</td>
<td>Ribonuclease H</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SERCA*</td>
<td>ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting (ATP2A1)</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIX5*</td>
<td>SIX homeobox 5</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>STAU1*</td>
<td>Staufen double-stranded RNA binding protein 1</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>tc-DNA</td>
<td>Tricyclo-DNA</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TRED</td>
<td>Trinucleotide repeat expansion disorder</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>vs</td>
<td>Versus</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>

*According to the HUGO Gene Nomenclature Committee guidelines (Wain, Bruford et al. 2002), gene names and symbols are written in *italics*. Same names are used to refer to mRNA, cDNA and proteins from the corresponding gene, but the protein name is not italicised. Human genes are written with all letters in uppercase, whereas mouse genes are written in lowercase but capitalized. In some cases (chapter 2), we additionally included the letter “h” or “m” preceding the gene name to further distinguish between human or mouse, respectively (e.g. *hDMPK* and *mDmpk*). The list of abbreviations only includes a selection of the genes, transcripts and proteins most relevant for this PhD thesis. For other names mentioned, a full description is given in the corresponding chapter when required.
CHAPTER 1

INTRODUCTION AND THESIS OUTLINE
| INTRODUCTION TO MYOTONIC DYSTROPHY |

Myotonic dystrophy (abbreviated DM for its Latin name: *dystrophia myotonica*) is a genetic, multisystem disorder described by a German physician (Hans Steinert) in a study published in 1909 [1], followed by another publication the same year by Batten and Gibb [2]. DM is the most common form of muscular dystrophy in adults, but disease symptoms are not limited to skeletal muscle, and include also neurological, cardiac, gastrointestinal, ocular, dermatological and endocrinal features [3,4]. There are two different forms of DM: type 1 and 2.

DM type 1 (DM1), also known as Steinert’s disease, is the most prevalent of the two [3]. The genetic cause was identified in 1992 as an expansion of a (CTG)n trinucleotide repeat sequence in the last exon of the dystrophia myotonica protein kinase (*DMPK*) gene on chromosome 19 [5-7]. However, soon after the discovery of the mutation, patients with DM1-like symptoms were identified that did not carry this triplet-repeat expansion. Immediately it became evident that these individuals belonged to families with myotonic dystrophy type 2 (DM2), a disorder characterized by slightly different, difficult to identify, myopathic problems. The genetic cause of this form of DM, also known as proximal myotonic myopathy (PROMM), was described in 2001 [8]. DM2 mutation was identified as an expanded (CCTG)n repeat in intron 1 of the gene for zinc finger protein 9 (*CNBP*) on chromosome 3 [9]. Interestingly, both the DM1 and the DM2 mutation are located in non-coding areas of the harboring gene: the 3’ UTR of *DMPK* mRNA and an intron of *CNBP* pre-mRNA, respectively.

Both disorders have very similar phenotypes, and differences in clinical manifestation are hard to distinguish: DM2 is generally milder and there is no congenital form of it like in DM1. Neurologic involvement is also milder in DM2 compared to DM1 [3,10,11] and different muscles and fiber types are preferentially affected in each DM type. DM1 patients mainly develop alterations in type 1 fibers of distal muscles, whereas DM2 patients experience most problems with type 2 fibers of proximal muscles [12,13].

The focus of my thesis will be on DM1, since most of the findings described here are based on models and specific treatments for this type of DM. Nevertheless, similar approaches may be potentially developed for DM2 [14].

**Epidemiology**

DM1 is most common in Europe or populations of European descent (3-15 patients per 100,000) [15], but is also present at lower prevalence in Japan (1 per 100,000), and even rarer in India [16-18]. Although several members of a family from the sub-Saharan region in Africa have been diagnosed with DM1 [19], the disease is practically absent in this continent [20]. In regions with special immigration and migration history, such as the Saguenay area in Quebec, the prevalence can be as high as 1 per 500 due to a founder effect [21]. Higher prevalence has also been reported in northern Sweden, the Basque region of Spain and Iceland (1 per 10,000) [3]. Prevalence numbers in different populations may even be slightly underestimated as older patients suffering mild DM1 symptoms remain frequently undiagnosed.
Chapter 1

## Symptoms

DM1 patients can present a highly variable clinical picture. So far, no consensus on DM1 clinical classification has been adopted but, depending on the severity and age of onset, different categories of disease manifestation can be distinguished [22,23].

### Late-onset myotonic dystrophy type 1

In this form of the disease, onset typically occurs after 40 years of age. It generally presents with mild weakness, possible myotonia (delayed relaxation of skeletal muscle after voluntary contraction or electrical stimulation) and cataracts at an early age (under 60). As mentioned in the previous section, not all patients in this category are properly diagnosed. Usually this happens only when a family member is diagnosed with DM1.

### Adult-onset/classical myotonic dystrophy type 1

This is the most common manifestation of DM1 [23]. Patients develop the first symptoms in the age interval between 20 and 40 years, which include skeletal muscle weakness (eventually leading to immobility), respiratory insufficiency, dysarthria and dysphagia [4]. Heart problems due to conduction defects and tachycardia are frequent and usually caused by fibrosis in the conduction system and sinoatrial node [24]. Together with cardiac complications, problems related to skeletal muscle weakness, frequently leading to respiratory failure [25], are the main cause of death in DM1.

Muscle weakness develops in facial, neck and distal limb muscles in parallel with muscle wasting. Atrophy of the temporal muscles and ptosis contribute to a characteristic myopathic facial appearance. Muscle fiber alterations in DM1 biopsies are found more frequently in distal muscles and include presence of central nuclei, sarcoplasmic masses, ring fibers and moderate atrophy of type 1 fibers in clinically weak muscles [3]. The most characteristic symptom of DM1 is myotonia, which is invariably present in the adult-onset form on both clinical examination and electromyography [3], but can occasionally be difficult to detect clinically.

DM1 patients may also develop neuropsychiatric problems. The most characteristic features are daytime sleepiness and apathy, involving a reduced perception and awareness of own disease symptoms and signs. Mild cognitive impairment is also frequently present [26,27]. Together, these changes may have an impact on cognition, learning, professional activity and sociologic aspects of the life of affected persons [28]. These neurological changes are normally accompanied by structural brain alterations, including abnormal white-matter changes, which can be detected by magnetic resonance imaging (MRI) [29].

A very frequent symptom is the development of cataracts, which are usually present much earlier than the average age in non-affected population. Although the specific type of cataract in DM1 (iridescent posterior subcapsular opacities) is almost pathognomonic of the disease [30], it is very often overlooked and surgically removed before proper identification. Retinal degenerative changes have also been reported [3,31].
The most frequent complaints from DM1 patients relate to gastrointestinal problems like constipation, diarrhea or incontinence [32]. In a disease advanced stage, dysphagia can become a challenge and lead to problems like aspirations and pneumonia [3,31].

DM1 patients suffer different endocrine alterations [3]. They are prone to develop insulin resistance and diabetes. Hypothyroidism is also common and can worsen the symptoms. Male patients frequently present hypogonadism and infertility. Miscarriages are a common feature in female patients.

Juvenile/infantile myotonic dystrophy type 1
In this form of DM1, cognitive and behavioral alterations start much earlier than muscle symptoms (before 10 years of age), causing intellectual disability early in the life of the patient [33]. Several psychiatric problems can be observed, including attention deficit disorder, anxiety, and mood disorder [34]. Approximately 50% of these children show intellectual impairment. Children do not suffer muscle weakness, wasting, or myotonia until an older age, which eventually causes physical symptoms similar to severe adult-onset DM1 [35,36].

Congenital myotonic dystrophy (CDM)
This is the most severe form of the disease, which is already present prenatally [37]. CDM can be detected in approximately 4% of all patients [23] and causes neonatal mortality in 18% of the infants [38]. However, these numbers could be higher in reality, because CDM may be incorrectly dismissed when the mother has not been diagnosed with DM1 and there is no prior history in the family [39]. It is characterized by reduced fetal movements, polyhydramnios, and various deformities [3,31]. At birth, babies have severe hypotonia caused by developmental defects (not by muscular degenerative changes), that can lead to respiratory failure and feeding difficulties [3,31].

Comparable to juvenile/infantile DM1, neurological alterations can be detected early (between birth and 10 years of age). Mental retardation and autism spectrum disorder are commonly seen in these patients [33]. During childhood, individuals with CDM exhibit delayed motor milestones that include oropharyngeal weakness, facial diplegia, marked dysarthria, and impairment of expressive communication [37]. Later on, between the age of 20 and 40, patients with CDM start to develop myotonia and other muscle symptoms characteristic of the classical form.

Genetics
DM1 is caused by expansion of an unstable (CTG)n trinucleotide repeat in the 3' untranslated region (UTR) of the DMPK gene, located on chromosome 19q13.3 [6,7]. In the non-affected population, the (CTG)n repeat length ranges between 5 and 37 triplets [31]. A repeat length between 37 and 50 is generally considered a “pre-mutation” [40], because individuals with a number of (CTG)n triplets in this range are asymptomatic but at risk of having children with larger, pathologically expanded repeats (due to an anticipation phenomenon, explained below). Full penetrance alleles occur with repeats greater than 50 CTGs and are, in most
cases, associated with disease [41]. However, many patients with a repeat size of 50-100 triplets are frequently underdiagnosed due to the fact that no or few mild symptoms (such as cataracts) appear at an older age. Individuals with a repeat size between 100 and 1000 triplets will inevitably develop either juvenile/infantile or adult onset DM1. Patients with more than ~1000 triplets (up to ~4000) normally suffer CDM, with severe symptoms present at birth, as detailed in the previous section. There is a certain correlation between (CTG)n length and age of onset and clinical severity [23], which is more reliable for repeat sizes in the range of 50 to ~500 [42]. Generally, patients with repeat sizes up to ~60 triplets develop first symptoms after the age of 60 and patients with less than 200-500 triplets are asymptomatic until adulthood or middle age [15].

As mentioned earlier, the DM1 mutation is unstable, with the length of the (CTG)n repeat changing size over time in different cells and tissues. Both expansion and contraction events may occur, but there is a strong tendency towards expansion of the newly generated alleles [43]. The instable behavior of the DMPK expansion is determined by interference of the DNA repair machinery with the topologically anomalous (CTG)n repeat track [44]. Failure to structurally repair the DNA across the mutation, possibly in combination with transcription-coupled events in DNA repair activity [45], determine the extent of instability. DNA replication itself seems to have no direct role, the DM1 repeat is actually more unstable in non-dividing cells [46,47].

Instability occurs at variable rates in different cells and tissues of the body, resulting in tissue heterogeneity and mosaicism of the repeat length. The level of repeat instability differs for example between cells in skeletal muscle, heart, brain and blood [48]. This phenomenon explains in part the broad spectrum of clinical manifestations seen in patients and the age-dependent and progressively worsening nature of the symptoms. It also has important implications for diagnosis and clinical management since the (CTG)n repeat in cells of the hematopoietic system is more stable [47]. Therefore, quantification of DM1 repeat length in blood is not a good predictor of the genotypic load in other tissues like muscle, where repeat length can increase at a higher rate as the patient ages [49]. Repeats of >37 CTG triplets are also unstable in the germline, causing the anticipation phenomenon by which the repeat expands between one generation and the next [50]. There are marked differences in repeat instability between spermatogenesis and oogenesis, being this phenomenon more active in the latter. Therefore, large genotypic jumps in DM1 most frequently happen when the disease allele is transmitted from the mother [51]. In fact, children with congenital DM1 are born almost exclusively from carrier mothers.

Interruptions of the (CTG)n repeat tract with other triplets, like CCG and CGG, usually at its 3’ end, have been reported in some patients [52]. Repeat imperfections are generally associated with modified phenotypic manifestations and reduced anticipation [53].

Pathology
DM1 is the first disease for which RNA was proposed as the initiator of pathology (RNA-mediated toxicity). As functions of protein products of the gene remain unaffected by the repeat mutation, being located outside the open reading frame, disease etiology
in DM is thus thought to be based on dominant negative effects of RNA-gain-of-function (Fig. 1). Until now this model is the most widely accepted as it accounts for the majority of symptoms present in DM1 and can also explain similarities with DM2 [54,55]. In fact, the mutations in both diseases are located in non-coding areas and there is no known functional relationship between the products of DMPK and CNBP. Model studies have demonstrated that transcription of expanded (C/CTG)n repeats into RNA are both necessary and sufficient to reproduce many aspects of the disease [4,56]. Expression of toxic RNA produces a variety of downstream alterations that can be broadly classified in two categories: (I) RNA splicing and processing alterations and (II) effects that compromise protein homeostasis and maintenance of cellular physiology. Below, I give a more extensive description of mechanisms associated with these two types of problems, but already here it is important to emphasize that it is almost impossible to distinguish between their primary and secondary causative effects on cell integrity and cellular function.

**Figure 1. DM1 molecular pathogenesis.** DM1 pathogenesis is complex and affects RNA and protein homeostasis. First, the long (CTG)n repeat in the mutant allele causes chromatin remodeling of the locus leading to reduced expression of the neighbouring gene SIX5 (A). Transcripts from the expanded DMPK gene form long (CUG)n hairpins that bind multiple proteins (e.g. MBNL, DDX5 and different types of hnRNP)s forming large ribonuclear inclusions (foci) that are retained in the nucleus. As a consequence, only wildtype DMPK transcripts will be translated, presumably causing DMPK haploinsufficiency (B). MBNL proteins are sequestered in foci reducing their steady-state levels (C). Toxicity exerted by the mRNA repeat expansion activates stress responses in the cell (D) leading to kinase activation and...
Chapter 1

DM1 splicing and RNA processing alterations

*DMPK* transcripts with a long (CUG)n repeat are retained in the cell nucleus, where they form long hairpin loops with C-G base pairs interrupted by U-U mismatches [57]. This type of structures tend to bind transcription factors and ribonucleoproteins (RNPs), forming aggregates in insoluble or diffuse/soluble state (named foci, after their detection by RNA FISH) [58]. *DMPK* foci abnormally bind RNPs, including members of the muscleblind-like family of proteins (MBNL1-3), hnRNP-H and DDX5 [59-61]. In turn, *DMPK* RNP foci cause stabilization of expression and activation of other proteins that are also involved in RNA metabolism, like CUG-binding protein 1 (CUGBP1, aka CELF1) [62] and Staufen1 (STAU1) [63,64]. These proteins function in various RNA processing and trafficking steps. Therefore, their alteration has serious consequences in trans for proper alternative splicing, polyadenylation, mRNA cellular distribution and miRNA expression [4].

Several of the mRNA processing alterations can be explained by diminished availability of MBNL proteins for normal cellular functions due to their sequestration and immobilization in ribonuclear foci [65-68]. Conversely, the steady-state concentration of CELF1 increases due to hyperphosphorylation and stabilization of the protein [69]. This posttranslational modification is most likely a secondary effect of repeat-mediated stress responses executed by different signaling kinases including protein kinase C (PKC). CELF1 and MBNL have antagonistic effects in pre-mRNA splicing, whereby the former factor normally acts as a splicing regulator that promotes production of isoforms that are typical for the embryonic developmental stage [70] and the latter factor promotes splice production of adult RNA isoforms [71]. The resulting effect of combined imbalance in these protein factors is aberrant splicing of pre-mRNAs from several genes [72], characterized by overexpression of embryonic splice isoforms in adult tissues [73-75].

RNA splicing alterations of multiple gene products have been studied, mostly in attempts where the authors tried to find direct associations with DM1 pathology. For example, aberrant splicing of chloride channel 1 (*CLCN1*) mRNA results in abnormal inclusion of exon 7a (normally only during embryogenesis), that abrogates expression of the protein by generation of a premature stop codon. Loss of *CLCN1* function has been identified as the cause of myotonia [76], the most characteristic symptom in DM1. Likewise, aberrant exclusion of exon 11 of the amphiphysin gene (*BIN1*), mediated by MBNL1 reduction, has been associated with central nuclei, developmental weakness and hypotonia in congenital DM1. Missplicing of calcium channels like *CaV1.1* and *SERCA* has been linked to muscle weakness and altered Ca\(^{2+}\) reuptake [77,78]. In addition, aberrant splicing of mRNAs for sarcomeric...
muscle proteins, such as the LIM domain binding 3 (encoded by LDB3), myomesin (MYOM1), myosin heavy chain 14 non-muscle (MYH14) and dystrophin (DMD) has also been noted in patients with DM1 [66,79-81]. Aberrant inclusion of exon 5 of cardiac troponin T (cTNT) may explain cardiac conduction defects [72]. Finally, alternative splicing of the insulin receptor (IR) can lead to expression of the insulin-insensitive form of this protein (due to exclusion of exon 11 in the mRNA), explaining insulin resistance in DM1 patients [82]. Taken combined, this evidence provides strong support for a model in which single events of missplicing are directly causal for distinct disease features of DM1. However, correlations between splicing abnormalities and pathobiological problems are not always that clear-cut, and evidence for a role of missplicing in DM pathology remains therefore circumstantial for most gene transcripts.

Perhaps not surprising, Mbnl1 knockout mice [83,84] and transgenic mice overexpressing CELF1 [85,86] (both lacking expanded toxic RNA expression) show widespread aberrant splicing. However, detailed phenotyping of these animal models revealed that consequences of abnormal splicing cannot always be directly translated to disease features as observed in DM1 patients [83,85,87]. For instance, although Mbnl1 knockout mice and transgenic mice expressing (CUG)n repeats from a transgene unrelated to DMPK (so-called HSA1R mice, see below) show similar patterns of missplicing observed in patients [66,75], both failed to recapitulate several features associated with DM1, like muscle wasting and weakness [56,83]. In addition, MBNL1 complementation in HSA1R mice prevented myotonia and restored adult-splicing patterns [88], but failed to rescue histological abnormalities, suggesting that muscle degeneration might not be due to MBNL1 loss alone. Therefore, other pathomechanisms than spliceopathy must be involved in the development of the disease.

Loss of cellular homeostasis

Nuclear retention of expanded DMPK transcripts is expected to lead to haploinsufficiency and reduction of DMPK protein levels, so the role of reduced DMPK expression in DM1 pathology should be also considered a possible contributing factor [89,90]. In fact, it should be noted that, although Dmpk-/- mice do not initially develop a characteristic DM1 phenotype, aged mice eventually did develop abnormalities [91-93]. Based on this, one would predict that a therapy that selectively silences the expanded allele would be safer for patients than total silencing of all DMPK gene products.

The (CTG)n expansion in the DMPK gene has been reported to suppress expression of the neighboring gene SIX5 (formerly known as DMAHP) by chromatin remodeling [94], a process that has been associated with cataract formation [95]. Methylation of CTCF sites flanking the (CTG)n repeat prevents binding of the CCCTC-binding factor and is related to antisense transcription of the DMPK, locus [96,97]. The transcription starts from the area of the SIX5 gene and reads through the DMPK sequence, including the repeat track. This phenomenon happens in all individuals, but the antisense transcript generated by DM1 patients contains an abnormally large (CAG)n repeat, which also forms stable hairpins. Antisense transcripts from the mutant DM1 locus may contribute to the imbalance in proteostasis by a process called repeat-associated non-ATG (RAN) translation [98]. This
phenomenon consists of the synthesis of peptides from the (CAG)n repeat, which is independent of the canonical AUG translation start codon in humans [99]. RAN translation of DMPK antisense transcripts gives rise to homopolymorphic peptides (e.g., poly-Q) in patient cells, including skeletal muscle cells, and in mouse models of the disease. Poly-Q peptides are central in the pathology of several neurodegenerative disorders [100]. However, what the contribution of RAN-translation products is to the overall DM1 pathology is currently still elusive [101].

Also global effects could be involved in creating imbalance in the DM1 proteome. A clear reduction in overall protein synthesis in muscles of DM1 patients has been reported [102]. This phenomenon is attributed to PKR activation upon its binding to the (CUG)n repeat (Fig. 1). PKR inactivates the eukaryotic translation initiation factor 2A (eIF2A) by phosphorylation at serine 51, which in turn reduces initiation of translation in the cell [103]. Importantly, PKR forms stress granules in cells expressing expanded (CUG)n transcripts and activates several cellular stress pathways [104], including the innate immune response and interferon signaling, which also may have a role in the development of cataracts. Prolonged activation of PKR and subsequent eIF2A inactivation also leads to stress in the endoplasmic reticulum (ER) in DM1 [105]. ER stress further blocks protein translation and eventually leads to apoptosis. Collectively, these ribostasis and proteostasis problems may be the principal pathophysiological contributors to the multisystemic phenotype in DM1 patients.

Animal models for RNA-based toxicology in DM1

The first animal model providing clear evidence for the toxic RNA gain-of-function hypothesis was the HSA18 mouse [56]. These mice carry a human transgenic insert composed of the actin alpha skeletal muscle (ACTA1) gene with its own promoter and a (CTG)n repeat of ~250 triplets in the 3' UTR (Table 1). Onset of transcription of this gene occurs relatively late in myogenesis and accumulation of transgenic (CUG)n-repeat RNA to high levels in this model is therefore not seen until late myofiber formation in vivo [106]. Then, abundant presence of (CUG)n-containing RNA leads to MBNL1 sequestration in ribonuclear foci and myotonic discharges. However, as also discussed in the last paragraph of section 1.4.1., the HSA18 model fails to recapitulate muscle wasting or weakness. Another disadvantage is that transgene expression is limited to skeletal muscle only.

Another mouse model developed for DM1 carries a cosmid-sized human transgenic insert spanning the entire expanded DMPK gene from a DM patient, including neighbouring genes DMWD and SIX5 plus all genomic cis elements that control temporal and spatial expression of DMPK [107]. The first generation of these mice, named DM300-328, contained an expansion of 300 CTG triplets (Table 1). Homozygous animals show mild multisystem features that include muscle histopathology, progressive muscle weakness, growth retardation and mild brain abnormalities [108]. Interestingly, this model also recapitulates the intergenerational instability of the mutation seen in patients [109]. Careful monitoring of repeat lengths over several generations of mice allowed the French researchers who developed this model to identify descendants with longer repeats, e.g., DM500 mice carrying 500 triplets, and DMSXL mice carrying 1000–1800 CTG triplets. Homozygous offspring of DMSXL mice present a more
severe phenotype, including splicing abnormalities in skeletal muscle, heart and CNS that are already present at one month of age [110].

Table 1. Information about the most relevant mouse models in DM1. DM300-328 mice contain the entire human DMPK locus with a repeat of 300-500 CTG triplets. DMSXL mice derived from the previous model by intergenerational repeat instability, leading to generation of mice with more than thousand triplets. HSA LR mice contain a repeat of ~250 CTG triples in the 3'-UTR of the ACTA gene, under control of the promoter of this gene. The phenotype, symptoms and original references of these models aresummarized in the table.

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Transgene/Mutation</th>
<th>Promoter/Expression</th>
<th>Phenotype/Symptoms</th>
<th>Original references</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM300-328</td>
<td>Human DM1 locus with ~300-500 CTG triplets</td>
<td>Human DMPK promoter/all DM1 related tissues (e.g., skeletal muscle, heart, CNS)</td>
<td>Homozygous mice: myopathy, reduced muscle strength, myotonia (generally very mild phenotype)</td>
<td>[107,108]</td>
</tr>
<tr>
<td>DMSXL</td>
<td>Human DM1 locus with ~1000-1800 CTG triplets (descendant of the DM300-328 line)</td>
<td>Human DMPK promoter/all DM1 related tissues (e.g., skeletal muscle, heart, CNS)</td>
<td>Homozygous mice: myopathy, reduced muscle strength, myotonia, reduced body size (more severe phenotype than DM500 mice)</td>
<td>[110]</td>
</tr>
<tr>
<td>HSA LR</td>
<td>Human ACTA1 gene with ~250 CTG triplets</td>
<td>Human ACTA1 promoter/ skeletal muscle only</td>
<td>Homozygous mice: strong myotonia, myopathy, no muscle weakness</td>
<td>[56]</td>
</tr>
</tbody>
</table>

Other mouse models that contributed to the elucidation of DM1 molecular pathogenesis (but are not used in this thesis) include DMPK and SIX5 knockout models [91,95], the Tg26 model overexpressing a (CTG)11 repeat in the DMPK gene [111], models for inducible expression of expanded CUG repeats [87,112], and models for MBNL inactivation [83,84] or CELF1 upregulation [85,86]. In addition, DM1 models that have been developed in other species like Drosophila [113], zebrafish [114] and Caenorhabditis elegans [115] can be useful for high-throughput screening of therapeutic compounds that alter repeat expansion behavior or RNA toxicity in vivo.

Biomarkers

In therapeutic screening studies, DM1 biomarkers are important as indicators of disease progression and possibly reversal, i.e. for monitoring the benefit of a therapeutic intervention. Not all biomarkers can be universally used, however. The choice of the right biomarker will depend strongly on the type of model that is under study, since, as described in the previous section, each model recapitulates different aspects of the disease. On the other hand,
when analyzing patient material, the source of the tissue (e.g. type of muscle biopsy) will also determine the analysis to follow. Moreover, for studies in vitro (with either cells derived from patients or from DM1 models, e.g. fibroblasts, myoblasts) one should also consider the limitations, as not all cell types or differentiation stages thereof will recapitulate every aspect of the corresponding adult tissue.

A broadly applicable and obvious method to assay the development of treatments aimed to reduce the load of toxic RNA in DM1 cells relies on the quantification of expanded DMPK transcripts, either present in the entire cell or in the fraction that is retained in the nucleus. Different techniques can be implemented to distinguish between products from normal and expanded alleles, like Northern blotting or allele-specific PCR assays.

Formation of ribonuclear inclusions (or foci) represent another biomarker that is frequently used. These foci can be detected in different amounts and shapes (diffuse/condensed) using FISH or MBNL immunostaining in cell nuclei of DM1 patients and models, both in vivo and in vitro [65]. This biomarker is useful not only to monitor treatments targeting the toxic RNA by silencing, but also treatments that aim to relieve toxicity by opening the hairpin structure and preventing abnormal MBNL binding [116].

A physiological readout for monitoring disease progression and severity in vivo is skeletal muscle myotonia. This symptom can be easily measured in patients by electromyography (EMG) and offers a good estimate of disease burden. For studies in DM1 animal models, it is important to consider that myotonia is only evident in HSA<sup>LR</sup> mice [56].

Finally, there are several characteristic spicing biomarkers in DM1 that can be measured [117]. Splice abnormalities that are most commonly used include CLCN1, SERCA, BIN1, DMD and MBNL1. Variation in alternative RNA splicing of these and other candidates can be tested in vivo and in vitro, and it is important to consider that different models and cells show distinct patterns of splicing abnormalities. Ideally, the characteristic DM1 splicing pattern in the model/cell type used should be determined in advance, as it has been done in some studies [118]. For instance, alternative splicing of exon 7a in CLCN1 is frequently used to monitor disease progression in muscle, but its application in vitro is more challenging because this gene is expressed late during myogenic differentiation, in stages that cannot be easily attained in cell culture. Furthermore, in addition to measuring an isoform change by RT-PCR, it is possible to monitor the correct location and functionality of the transcript isoform products. For instance, following the CLCN1 example, it is common to study the expression and location of CLCN1 proteins in the membrane of muscle fibers by immunohistochemistry.

**Treatment prospects**

**Symptomatic treatment and care**

Currently, there is no approved treatment to eliminate the root cause of DM1 or its direct downstream effects. Clinical efforts can only be taken to manage symptoms and minimize disability by palliative care. For instance, mobility problems are counteracted using canes, braces, walkers or scooters. Cardiac and respiratory functions should be always monitored longitudinally to apply the corresponding treatment if necessary (e.g. using a pacemaker or defibrillator). As discussed above, cataracts are frequently removed surgically. Excessive
daytime sleepiness can be improved using methylphenidate or modafinil. Gastrointestinal complications can also be treated with medication. All these measures can contribute to alleviate symptomatology but, given the progressively worsening nature of DM1 pathology, they are inefficient to provide a sustained relieve of disease burden and complications for patients and relatives. Therapies to treat myotonia are currently undergoing clinical trials (ClinicalTrials.gov Identifier: NCT01406873), involving the use of Na\(^+\) channel blockers like mexiletine [119].

**Development of molecular therapies**

Multiple approaches aiming to neutralize toxic DMPK RNA to stop progression of disease are under development [120]. There are two possible ways to achieve this: by silencing/degradation of (expanded) DMPK transcripts or by blocking the associated toxic effects (i.e. by interfering with protein binding and foci formation).

The first ever developed strategy for RNA targeting in DM1 made use of ribozymes, RNA molecules with catalytic activity, to reduce the amount of (CUG)\(_n\) triplets in the cell by modulation of splicing events [121,122] or induction of an endonucleolytic cleavage [123].

RNA interference (RNAi) [124,125] with transient inhibition by short RNAs or permanent expression of shRNA (small hairpin RNA), has been also applied to silence DMPK transcripts. For example, DMPK mRNA was silenced by lentiviral vectors that delivered shRNAs targeting the 5’ end of the mRNA, resulting in reduction of both normal and expanded transcripts [126]. Others used siRNAs targeting the (CUG)\(_n\) repeat itself, leading to reduced levels of toxic RNA in HSA\(^{LR}\) mice [127]. More recently, another study used adeno-associated virus to deliver RNAi hairpins systemically to target the (CUG)\(_n\) repeat in HSA\(^{LR}\) mice, leading to a reduction of toxic RNA, myotonia inhibition and splicing correction [128].

Other therapeutic strategies make use of small organic molecules or peptides that bind the expanded (CUG)\(_n\) repeat and displace sequestered MBNL proteins, thus disrupting nuclear foci. Pentamidine was the first molecule identified with this role [129], although the exact mechanism of action is not clear. Other studies focused on the specific design of chemically synthesized aminoglycosides based on compounds that bind RNA loops [130], resulting in the discovery of modularly assembled ligands that bind (CUG)\(_n\) repeats with high affinity. Finally, from large screenings in Drosophila, other promising (CUG)\(_n\)-binding compounds have been identified, like the peptide ABP1 as a recent example, which also showed beneficial effects in a mouse model [116].

Antisense oligonucleotide strategies are currently the most extensively studied and promising approaches to treat DM1 [131]. Since this was the strategy investigated during my Ph.D. research, it will receive special attention and will be discussed in detail in the next section.
Chapter 1

INTRODUCTION TO ANTISENSE OLIGONUCLEOTIDES

AONs are single-stranded, short nucleotide sequences complementary to RNA or DNA targets in the cell. They were applied for the first time in cell culture by Zamecnik and Stephenson in 1978 to inhibit viral replication [132]. Since then, many other applications have been developed, because AONs can be potentially used to modulate expression of virtually any gene or transcript in the cell.

Mechanisms of action

There are several mechanisms of RNA modulation in antisense technology [133]. Most AONs interact or block certain cellular pathways involved in RNA biology (Fig. 2). They can recruit endogenous enzymes and factors to modulate expression of the intended RNA target. The most relevant mechanisms that are currently being employed include:

Modulation of pre-mRNA processing

AONs can alter pre-mRNA splicing by either blocking donor or acceptor splice sites or splicing enhancer or silencer sequences [134]. Upon binding, AONs sterically block access to protein factors or ribonucleoprotein complexes involved in pre-mRNA splicing. Therefore, this approach can be used to induce exon skipping or promote exon inclusion of an alternatively spliced exon. In addition, AONs can be designed to interfere with poly(A) site selection in a transcript [135].

Translation inhibition

AONs can be directed towards the translation initiation site of a given mRNA to specifically block ribosomal assembly and protein synthesis [136]. The application of this strategy is challenging due to the limited sequence stretch of mRNA available to design AONs that can bind in this region and efficiently block the process.

RNase-H recruitment

RNase H is involved in DNA repair and the removal of Okazaki fragments during DNA replication. This enzyme binds to RNA-DNA heteroduplexes and cleaves the RNA strand, leaving an unprotected 5'-phosphate end and releasing the DNA strand intact [137]. RNase H is represented by a family of enzymes present in all mammalian cells. When using an AON of five or more consecutive DNA nucleotides, this enzyme mediates cleavage of the RNA in the RNA-AON heteroduplex [138]. Since RNase H is more abundant in the cell nucleus, silencing of RNAs in this compartment by this mechanism has been shown to be more effective [139].

RNA interference

Careful design of AONs to mimic the activity of miRNAs is possible, but certain requirements in nucleotide chemistry have to be met in order to obtain successful recognition of the AON by the RNAi machinery [140-143]. AONs with this property have also been termed
Introduction to antisense oligonucleotides

Single-stranded siRNAs (ss-siRNAs). They successfully enter the RNAi pathway, being directly recruited by RISC downstream of Dicer processing [144]. On the other hand, AONs can also be used to interfere with the activity of miRNAs. These AONs, usually termed antimiRs or antagonirs, function as competitive inhibitors of miRNAs, blocking their ability to bind target mRNAs [145,146].

Ribozymes and other RNA-silencing strategies

Other approaches developed to induce selective RNA degradation include ribozymes and DNAzymes, well-structured oligonucleotide-based molecules that possess inherent catalytic activity as nucleases [147-149]. In addition, other nucleases and natural RNA-degrading pathways present in the cell can be potentially used to promote specific RNA knock-down [150]. For instance, nonsense-mediated decay can be triggered with AONs that generate premature stop codons by aberrant exon skipping [151]. Finally, the fast growing collection of CRISPR/Cas type of nucleases may be used in the future to target RNA for degradation in living cells [152].

Figure 2. Molecular mechanisms of RNA modulation by antisense oligonucleotides. An antisense oligonucleotide (AON) can modulate the fate of (pre-)mRNA by multiple mechanisms. They can influence pre-mRNA processing, like polyadenylation or splicing. Exon skipping can be achieved by blocking exonic splicing enhancers or donor/acceptor splice sites. Skipping of an in-frame exon can be used to remove mutations, but results in a shorter mRNA and protein. In contrast, when the skipped exon is out-of-frame, a premature stop codon is generated, which generally leads to transcript degradation by nonsense mediated decay (NMD). AONs can also be designed to block translation or to silence gene expression by several other mechanisms, for instance, gapmer-type of AONs can recruit RNase H, an enzyme that is mainly expressed in the nucleus. As such, gapmers can also be directed...
Chapter 1

AON chemistry

Chemical modification of an AON is necessary to modulate its fate in vitro and in vivo. Unmodified oligonucleotides undergo fast degradation by endogenous nucleases present in the cell or biological fluids. Moreover, when administered in vivo, circulating DNA and RNA are rapidly removed by liver and kidney [153]. Together, plasma nucleases and excretion produce an estimated half-life of ~15 minutes in serum [154], a time that can be significantly extended by changing the nucleotide chemical composition (see below). In addition to increase stability and improve pharmacokinetics, AON modifications can be used to enhance RNA binding affinity. These aspects are of importance for successful application of AONs as therapeutic agents in medical applications [133,155].

Figure 3. Some chemical modifications applied in antisense technology. The first generation of AONs consisted of the introduction of phosphorothioate (PS) internucleoside linkages by replacing an oxygen atom by a sulfur in the phosphate backbone. In a second generation of modifications, alkylation of the 2' position of the ribose was implemented to further improve nuclease resistance while increasing binding affinity. A vast amount of chemical modifications in the sugar pentose or overall nucleotide structure have been implemented in a third generation of AONs (only the most relevant examples are depicted in this figure). PS internucleoside linkages are usually combined with those chemical modifications that maintain the phosphate backbone, but is not possible in PMO and PNA. Arrows point to specific chemical modifications in the nucleotide structure. B: Nitrogenous base.

*Relative order of some types of nucleotide modifications according to their approximate change in Tm compared to unmodified DNA. BNA: bridged nucleic acid (ENA, LNA and cEt).
The nucleotide structure can be divided in three parts (Fig. 3), all amenable to manipulation: internucleoside linkage (phosphate backbone), pentose sugar and nitrogenous base. To date, a vast collection of chemical modifications has been implemented in successive generations of antisense technology [156]. According to the part of the nucleotide that is modified, they can be broadly classified in four groups:

### Modifications in the internucleoside linkage

Eckstein and colleagues generated in 1969 the first generation of AON modifications [157], which consisted of the introduction of a phosphorothioate (PS) modification of the nucleotide backbone. In this class of AONs, one of the non-bridging oxygen atoms in the phosphodiester backbone is replaced with a sulfur (Fig. 3) [158]. This modification notably improved nuclease resistance and pharmacokinetic properties, increasing the half-life in serum to approximately 9 hours [159] and resulting in improved biodistribution and pharmacokinetics [160]. PS AONs are characterized by non-specific binding to proteins, especially those that interact with polyanions [161]. Binding to plasma proteins and cells allow these AONs to be retained in the body for longer periods [162]. However, PS oligodeoxynucleotides have a somewhat reduced binding affinity to RNA compared to unmodified DNA, with an approximate melting temperature (Tm) reduction of 0.5 °C per linkage [163,164].

### Pentose sugar modifications

After the introduction of the PS linkage, research focused on improving binding affinity to RNA. As RNA/RNA duplexes are more stable (i.e. have a higher Tm) than DNA/RNA duplexes, introduction of RNA-type nucleotides into DNA type AONs was found to increase affinity towards complementary RNA targets [155]. The most commonly used modification involves changes at the 2’ position of the pentose sugar (Fig. 3), very frequently combined with a PS backbone.

AONs consisting of full-length 2’-modified RNA are not able to recruit RNase H because this enzyme only recognizes DNA-RNA hybrids (see above). To induce RNase H-mediated degradation of target RNA, it is possible though to design a gapmer type of AON, which possesses a central gap of PS DNA to enable formation of a RNase H-recruiting DNA/RNA duplex, flanked by 2’-modified RNA ‘wings’ which enhance affinity and confer nuclease resistance for the entire AON.

The most relevant examples of pentose-modified AONs include:

#### 2’-O-alkyl modifications

2’-O-alkyl modifications were developed after the introduction of the PS backbone and, as such, they are very frequently classified as the second generation of AON modifications [155]. They include an O-alkyl group at the 2’ position of the ribose sugar. The two most important modifications that are frequently applied in antisense technology involve the introduction of 2’-O-methyl (2’-OMe) and 2’-O-methoxyethyl (2’-MOE) groups [165]. These modifications confer increased binding to RNA by reducing the flexibility of the furanose ring, resulting in a more stable hybridization.
Chapter 1

Bridged nucleic acids (BNAs)
BNAs consist of ribonucleotides in which the 2’-oxygen of the ribose is linked to the 4’-carbon (Fig. 3). The most relevant BNAs are: ethylene-bridged nucleic acid (ENA) [166], locked nucleic acid (LNA) [167] and constrained-ethyl nucleic acid (cEt) [168,169]. ENAs contain an ethylene bridge, whereas LNA and cEt contain a methylene bridge. BNAs confer enhanced stability against nucleolytic degradation [170], and an even further increased binding affinity to RNA, due to a more reduced flexibility of the furanose ring over 2’-substituted nucleotides. They provide an increase in Tm of 5-10°C per nucleotide when introduced into unmodified DNA [171,172]. This feature makes BNAs ideally suited when strong binding to the target RNA is desired. Caution should be taken however, when trying to introduce many BNAs in an AON: inducing a too-high Tm may well lead to loss of mismatch discrimination and thus cause synthesis issues (aggregation) and/or off-target effects (binding to unintended sequences). Thus, combining a few BNA residues with other type of chemical modifications is normally the approach of choice to generate gapmers or mixmers. On the other hand, BNAs can be very helpful when short AON sequences are required, like those designed to block miRNAs [145].

2’-Fluoro-2’-deoxy nucleic acid (2’-F) and 2’-F arabinosyl nucleic acid (FANA)
2’-F and FANA are characterized by a fluorine substituent on the 2’ position of the pentose ring (Fig. 3). In 2’-F RNA, this confers increased binding affinity, to between that of 2’-O-alkyl RNA and LNA/ENA. In FANA, an arabinose sugar (the 2’ epimer of ribose) is used instead to generate the oligonucleotide. AONs with FANA chemistry are recognized by RNase H and are therefore able to induce cleavage of target RNA by this mechanism [173].

Tricyclo-DNA (tc-DNA)
This chemical modification is derived from DNA by addition of three carbon atoms between the 5’ and 3’ position of the ribose (Fig. 3). This type of conformationally constrained DNA analog shows enhanced binding affinity to RNA and nuclease resistance, but it is not able to recruit RNase H [174]. Tc-DNA has been shown to possess interesting pharmacokinetic and tissue uptake properties [175].

Alternative scaffolds
This group is formed by AONs in which both the phosphate backbone and pentose sugar have been completely replaced by other structures. As such, the introduction of a PS modification is not possible, since phosphate linkages are not present in this type of AONs. Two relevant examples of these modifications are described below.

Phosphorodiamidate morpholino oligomer (PMO)
These AONs are nonionic nucleotide analogs with a backbone of phosphorodiamidate linkages instead of phosphodiester/phosphorothioate bonds, and morpholine-derived scaffolds instead of ribose-derived ones (Fig. 3) [176]. These AONs (often called morpholinos) are frequently used in developmental and functional gene studies in zebrafish [177]. Because
their backbone is uncharged, PMOs do not interact with proteins and have reduced cellular uptake properties compared to other chemistries. Their binding affinity to RNA is higher than unmodified DNA but lower than second generation modifications.

**Peptide nucleic acids (PNAs)**

PNAs are composed of repeating \(N\)-(2-aminoethyl)-glycine units connected by amide (peptide) bonds with bases linked by a carboxymethylene moiety (Fig. 3) [178]. As such, PNAs are depicted as peptides, with the N- and C-termini corresponding to the conventional 5′/3′ denomination, respectively. PNAs can bind complementary sequences in both antiparallel and parallel orientation [179] and are able to invade duplex DNA with high affinity and specificity [180,181].

**Nitrogenous base modifications**

Nitrogenous bases are responsible for nucleic acid hybridization by conventional Watson-Crick hydrogen bonds. The nitrogenous bases are adenine (A), guanine (G), cytosine (C), thymine (T, only present in DNA), and uracil (U, only present in RNA). Several modifications can be applied that are sometimes also present in nature, like the introduction of a 5-methyl group in a cytosine (as present in natural DNA via epigenetic modifications), or the replacement by non-conventional bases. For instance, the introduction of inosine (I, naturally present in RNA by post-transcriptional modifications) [182], can be used to expand the hybridization possibilities since it can bind to adenine, uracil and cytosine.

**AON delivery**

**Pharmacokinetics (PK)**

Oral administration is generally ineffective because the physicochemical properties of AONs impede their gastrointestinal absorption. These properties include their relatively large size/molecular weight (~5-10 kDa) and their hydrophilic nature [183]. Therefore, AONs are most frequently administered by subcutaneous or intravenous routes *in vivo*. As discussed above, AONs with PS backbone have improved PK characteristics compared to their PO analogs or uncharged AONs, like PNAs and PMOs, which are rapidly excreted with the urine [184,185]. This advantage is due to PS binding to serum proteins, something that delays the process of glomerular filtration, resulting in improved tissue distribution and bioavailability [133]. Levels of PS-modified AONs in circulation decline during the first hours post-administration because they are rapidly transferred from blood into tissues. Accumulation occurs mainly in the liver parenchyma and proximal epithelium of renal cortex, but there is some functional uptake in most other tissues, except the central nervous system (CNS). In addition, after reaching peripheral organs, they can reside for long periods of time (weeks to months) [186].

Specific delivery into the CNS is very challenging because AONs do not cross the blood-brain barrier easily [187]. Therefore, the potential application of antisense technology to treat neurological disorders has to overcome this limitation using alternative administration routes, like intracerebroventricular (i.c.v.) infusion. AONs inoculated into the cerebrospinal fluid show remarkable broad distribution through the CNS [188,189].
This has important implications for the clinic, since diseases affecting brain can be potentially treated with AONs using less invasive administration methods than i.c.v., like intrathecal injections.

Cellular uptake
A challenge for successful application of an AON treatment is the delivery of these molecules to their targets in the relevant compartment inside the cell. Several studies have focused on understanding the mechanisms behind free-AON uptake and intracellular trafficking [190]. Currently, it is commonly accepted that endocytosis and fluid phase pinocytosis are the major mechanisms of oligonucleotide internalization [191]. Most of the AONs accumulate in the lysosomal compartment over time, where they can reside for long periods of time due to their intrinsic resistance to cellular nucleases [192]. However, in the course of vesicular trafficking, a small fraction of AON is believed to escape to reach the functional target in the cytosol or the nucleus [193].

Antisense strategies for DM1
AONs currently developed for DM1 can be broadly divided in two groups: those that target the expanded (CUG)n repeat tract in DMPK mRNA and those that are specifically designed for binding to unique target sequences elsewhere in the transcript.

AONs that target the (CUG)n repeat in DMPK mRNA
This category of AONs can be used to displace MBNL proteins sequestered by the hairpin and/or to induce preferential degradation of expanded DMPK RNAs. Interestingly, two independent studies using AONs with blocking chemistries (non RNase H-recruiting) found that this type of strategy also leads to degradation of expanded (CUG)n mRNA. In both cases, transcripts from the expanded allele were silenced to a large extent, possibly since more AONs were able to bind to these transcripts than to RNAs from the healthy allele. In the first study that was published on this topic, Wheeler et al. [194] described the use of a 25 nt AON with PMO chemistry and (AGC)8A sequence named CAG25. In test tube experiments, CAG25 was able to invade (CUG)n hairpins to release MBNL1 protein and prevent it from binding. Treatment of HSA18 mice by intramuscular injections resulted in reduction of nuclear foci, redistribution of Mbnl1 protein and normalization of aberrant splicing, restoring Clcn1 protein expression and minimizing myotonia. Contrary to expectation, CAG25 administration also resulted in ~50% reduction of expression from the transgene, while other transcripts with short (CUG)n repeat remained unaffected [194].

In the second study on (CUG)n-specific AONs, our own laboratory identified a 2'-OMe AON of (CAG)7 sequence, called PS58, as a promising therapeutic agent for DM1 [195]. PS58 reduced expanded DMPK transcript levels in a DM1 mouse cell model and in patient myoblast cultures. DMPK mRNA bearing a repeat of normal size and other transcripts with short (CUG)n repeats were left essentially intact. Findings were corroborated in DM300-328 and HSA18 mice by intramuscular administration, which resulted in ~50% reduction of
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(CUG)n RNA in both models. In the HSA\textsuperscript{LR} model, nuclear foci were reduced and DM1 splicing abnormalities were moderately improved [195].

More recently, an approach by which an engineered hU7-snRNA containing a poly-CAG antisense sequence was delivered by an adeno-associated virus (AAV) also resulted in specific degradation of expanded DMPK transcripts via an unknown mechanism [196].

The RNA silencing mechanism of the blocking antisense strategies directed to the (CUG)n repeat is currently unknown. This topic will be discussed further in this thesis, since much experimental effort was put in elucidating this pathway.

Other researchers in the DM1 field subsequently used LNA and MOE gapmers targeting (CUG)n repeats [197]. They found that these AONs are able to silence expanded DMPK transcripts \textit{in vivo}, after local administration in muscle of a DM1 mouse model. In addition, combined administration of gapmers with CAG25 AON (described above), further enhanced silencing activity. Interestingly, small fully-modified LNA AONs complementary to the repeat displaced MBNL proteins from the hairpin and corrected foci and alternative splicing defects without inducing degradation of expanded DMPK transcripts [118].

**AONs that target DMPK transcripts outside the repeat**

The disadvantage of strategies aimed to silence DMPK mRNA via binding to unique sequences outside the expanded repeat is that both normal and expanded DMPK transcripts might be blocked or degraded, so detrimental effects of total DMPK knock-down could become a concern. Normal roles of the DMPK gene that have been described include remodeling of the actomyosin cytoskeleton [198], maintaining nuclear envelop integrity [199] and preventing ROS-induced damage [199]. As discussed above, complete lack of DMPK protein function, as achieved in Dmpk knockout mice, has no direct overt phenotypic consequences, but eventually abnormalities do develop [91-93]. Thus loss of DMPK function could be a point of future concern, but possible problems from expression of repeat-carrying RNA may outweigh the problems associated with total DMPK RNA loss. We think that arguments of this kind must have pushed developments further, and currently complete DMPK mRNA breakdown strategies have already entered the stage of clinical trials, testing safety, tolerability and efficacy of IONIS-DMPK\textsubscript{Rx} in DM1 patients (ClinicalTrials.gov Identifier: NCT02312011).

One of the first preclinical studies of this kind employed 2′-MOE gapmers to target transgenic ACTA1 RNA in HSA\textsuperscript{LR} mice outside the repeat region [139]. These AONs showed long-lasting silencing capabilities after systemic administration, especially for gapmers binding downstream to the repeat. Transgenic RNA reduction and correction of Serca splicing, myotonia and central nuclei were still apparent one year after the treatment. Furthermore, gapmers targeting the 3′ UTR of DMPK RNA at unique sequences could also silence these transcripts after systemic administration in another DM1 model [139]. The authors of this study claim that RNase H can induce an efficient and continuous cleavage of the mRNA target with only small amounts of gapmers present in the nucleus.

Finally, a recent study characterized the effect of systemic administration of a gapmer that targets mouse, monkey and human DMPK mRNA outside the repeat [200]. This AON,
containing 2'-4' constrained ethyl (cEt) modifications, showed silencing activity against DMPK RNA in muscles of wildtype mice, DM1 mouse models and cynomolgus monkeys.

Other AONs in the clinic

The first antisense drug approved for commercialization was fomivirsen, a fully-modified PS AON that was registered in 1998 as a treatment for cytomegalovirus-induced retinitis in immunocompromised patients with AIDS [201]. This AON was delivered via intraocular injection and reached efficiently the target site (retina), something that contributed to the success of the drug. Since then, many other antisense strategies have been tested in the clinic with only limited success [202]. Recently, mipomersen (Kynamro®), a 2'-MOE gapmer with 5-methyl cytosines targeting the mRNA of apolipoprotein B in the liver [203,204], was approved for treatment of familial hypercholesterolemia.

Despite their slow entry on the market, AONs still represent a drug platform with high therapeutic potential if ways to overcome the biological barriers towards improved delivery are developed. Given the central role of RNA in molecular biology [205], this technology could be used to interfere with molecular pathomechanisms in many disorders [133]. It is therefore not surprising that there is a broad spectrum of diseases for which AONs are currently in development, including β-thalassemia [206], hypertriglyceridemia [207], Duchenne muscular dystrophy (DMD), Huntington’s disease (HD), spinal muscular atrophy [208], amyotrophic lateral sclerosis [209], infectious diseases [210], inflammatory conditions [211] and cancer [212]. Since it would be impossible to cover them all here, only two examples with clear relevance for the DM1 field will be given. First, I will describe developments in DMD, a neuromuscular disorder currently undergoing clinical trials in which AONs are tested. Secondly, I will focus on HD as an example of a disease caused by a trinucleotide repeat expansion.

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is the most common lethal X-linked disease in man, affecting 1 in 3500 male births [213]. DMD is associated with severe, progressive muscle weakness and typically leads to death between the ages of 20 to 35 years. Other symptoms include poor motor skills, progressive difficulty moving, fatigue and higher risk of neurobehavioral disorders. Patients are frequently wheelchair-bound before the age of 12 and need respiratory support by the second decade of their life. DMD is caused by lack of dystrophin expression. This protein forms a multi-subunit complex, the dystrophinglycoprotein complex (DGC), responsible for connecting the cytoskeleton of muscle fibers to the extracellular basal lamina [214]. In absence of dystrophin, the DGC structure is disrupted, affecting the membrane stability of muscle fibers [215], eventually leading to cell death and replacement by connective tissue. Despite extensive efforts, there is no treatment available for DMD patients yet.

Dystrophin loss in DMD patients is caused by mutations in the DMD gene at locus Xp21 [216]. Deletions, duplications or point mutations in this gene result in a frame shift in the open reading frame (ORF) in the transcript [217], often creating a premature stop
Introduction to antisense oligonucleotides

codon that abrogates functional dystrophin protein synthesis. In an approach to restore the ORF of DMD transcripts, AONs targeting exon splicing enhancer (ESE) sequences in exons containing DMD mutations have been tested with promising results [218]. These AONs recover dystrophin expression by generating a shorter but functional transcript by exon skipping. Two types of AON chemistries using this strategy have been evaluated clinically: 2’-OMe PS [219] and PMO [220].

Huntington’s disease

Huntington’s disease (HD) is a devastating inherited neurodegenerative disease caused by an expansion of a (CAG)n repeat in exon 1 of the huntingin (HTT) gene, located in chromosome 4p16. The (CAG)n repeat is present in the ORF of the gene and it codes for a polyglutamine (poly-Q) tract located at the N-terminus of the HTT protein, ubiquitously expressed throughout the body and brain [221]. HD is characterized by progressive motor and cognitive decline, leading to dementia and other psychiatric manifestations like depression and psychosis. Generally, first symptoms occur in midlife, eventually leading to premature death within 15-20 years after disease onset [222]. Together with DM1, it is one of the most well-known trinucleotide repeat disorders and today still remains incurable and insufficiently understood. HD has a prevalence of 5-10 per 100,000 in populations of European descent [223]. Similarly to DM1, repeat length is correlated to age of onset and disease symptoms, starting with 36 or more CAG triplets [224]. Above this threshold, it confers a neurotoxic gain-of-function to the mutant protein [225], producing intracellular aggregates and leading to progressive neuronal loss in several brain regions, predominantly in the striatum [226].

As in DM1, the most promising antisense therapies in development for HD aim to reduce the amount of mutant HTT RNA (and protein) [227]. Also in this case, two possible strategies using AONs are in (pre)clinical development: (a) targeting the expanded trinucleotide repeat, resulting in preferential silencing of RNA products from the expanded allele [228], or (b) targeting the HTT transcript outside the repeat, which can also be designed to be allelespecific in certain subpopulations of patients by using SNPs [229]. There is overall consensus that achieving silencing selectivity for products of the mutant allele would be preferable, given the important and diverse cellular functions of the wildtype HTT protein, including normal development, function and maintenance of neurons [230,231]. Nevertheless, the first clinical study testing an antisense strategy for HD uses an AON (IONIS-HTTRx) to reduce levels of both wildtype and mutant HTT transcripts. The trial has recently started and will evaluate the safety, tolerability, pharmacokinetics and pharmacodynamics of AONs administered in HD patients by intrathecal injections (ClinicalTrials.gov Identifier: NCT02519036).
Chapter 1

| THESIS OUTLINE |

The collection of experiments described in this thesis aim to elucidate aspects of antisense oligonucleotide (AON) treatment for neuromuscular diseases, focusing especially on their application to myotonic dystrophy type 1 (DM1). Chapter 1 serves as a general introduction of this book. This chapter is divided in two sections: the first part describes several aspects of DM1, such as epidemiology, symptoms, genetics, pathogenesis and care. The second section introduces AONs, giving a description of their development and applications, and a discussion on why they represent a promising therapeutic approach for DM1. Other aspects, such as in vivo distribution and cellular uptake are also considered.

During my research, I mainly focused on the development of AONs that make use of the most important polymorphism in DM1: the (CTG)n repeat in the DMPK gene. RNA products of this gene, when expanded above a certain threshold, represent the root cause of the disease. We tested a battery of (CAG)n AONs of different lengths and chemistries and studied their silencing effect on expanded DMPK transcripts, comparing our data to other approaches being currently developed. These experiments are presented and discussed in Chapter 2. Important parameters for preclinical development, such as allele selectivity, immunogenicity, stability, intracellular localization and potential effects on unrelated transcripts bearing (CUG)n or (CAG)n repeats were also studied. Additionally, based on some experimental evidence of this chapter and previous knowledge, a discussion about the silencing mechanism of AONs targeting mRNA trinucleotide repeats is started in this chapter. This topic is further extended in Chapter 3, where AON-RNA interactions with cytoplasmic and nuclear proteins of a DM1 model were studied in vitro. We implemented a pull-down method to isolate AONs hybridizing to a (CUG)n-containing RNA and studied proteins that interact with this duplex. Some of the identified proteins have molecular roles potentially related to the mechanism, such as nuclease and RNA helicase activity.

In Chapter 4, I present the analysis of transcriptome changes after treatment with (CAG)n and control AONs in cells from unaffected individuals and patients suffering from DM1. These experiments were performed to monitor changes in expression pattern upon AON treatment. Some of these changes might respond to the normalization of the genetic disease signature upon silencing of the corresponding mutation (pathologic reversal) and could give very valuable information on how fast altered molecular features could be corrected. On the other hand, other changes may be due to unspecific response of the cells to triplet repeat AONs, and their study might help to predict side effects of the treatment. In order to discriminate changes responding to pathologic reversal or side effects, an extensive and careful analysis of the data (based on RNA-Seq and SAGE) was performed using several bioinformatics tools.

The main challenge for a therapeutic approach with AONs is improving intracellular localization within cells of targeted tissues after systemic treatment in vivo. In some disease conditions, such as DMD, affected cells suffer from loss of membrane integrity, which in turn facilitates uptake of large compounds like AONs. Essentially nothing was known on membrane integrity in DM1, as this had never been addressed in detail. Information on this topic in DM1 will be useful to anticipate the scope of the treatment and design of
adequate delivery strategies. I deal with this subject in Chapter 5, where we performed an exhaustive analysis of membrane integrity in muscles and other tissues of DM1 mouse models and patients and related our findings to several in vivo studies with AONs that were performed by our group and others. Since results of Chapter 5 clearly show that cell membranes in DM1 do not have affected integrity (being indistinguishable from healthy controls in terms of permeability) new studies investigating mechanisms of AON uptake and intracellular distribution in DM1 are important. Therefore, we next investigated how AONs of different chemistries, including AONs conjugated to a muscle-homing peptide, are internalized by DM1 myoblasts and myotubes. These experiments are presented and discussed in Chapter 6. Free uptake incubation of AONs (so-called gymnosis), intracellular vesicular trafficking and nuclear delivery and activity were studied by microscopy combined with molecular biological analysis of the fate of the RNA targets. Effects of interference with cellular import and trafficking mechanisms were also investigated in this chapter. Finally, the results presented in this thesis are summarized, discussed and put into perspective in Chapter 7.
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CHAPTER 2

DESIGN AND ANALYSIS OF EFFECTS OF TRIPLET REPEAT OLIGONUCLEOTIDES IN CELL MODELS FOR MYOTONIC DYSTROPHY

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Chapter 2

ABSTRACT

Myotonic dystrophy type 1 (DM1) is caused by DM protein kinase (DMPK) transcripts containing an expanded (CUG)n repeat. Antisense-oligonucleotide-(AON)-mediated suppression of these mutant RNAs is considered a promising therapeutic strategy for this severe disorder. Earlier, we identified a 2’-O-methyl phosphorothioate-modified (CAG)7 oligo (PS58), which selectively silences mutant DMPK transcripts through recognition of the abnormally long (CUG)n tract. We present here a comprehensive collection of triplet repeat AONs and found that oligo length and nucleotide chemistry are important determinants for activity. For significant reduction of expanded DMPK mRNAs a minimal length of five triplets was required. 2’-O,4’-C-ethylene-bridged nucleic-acid-(ENA)-modified AONs appeared not effective, probably due to lack of nuclear internalization. Selectivity for products from the expanded DMPK allele in patient myoblasts, an important requirement to minimize unwanted side effects, appeared also dependent on AON chemistry. In particular, RNase-H dependent (CAG)n AONs did not show (CUG)n-length specificity. We provide evidence that degradation of long DMPK transcripts induced by PS58-type AONs is an RNase-H independent process, does not involve oligo-intrinsic RNase activity nor does it interfere with splicing of DMPK transcripts. Our collection of triplet-repeat AONs forms an important resource for further development of a safe therapy for DM1 and other unstable microsatellite diseases.
INTRODUCTION

Myotonic dystrophy type 1 (DM1) is an autosomal dominant, trinucleotide repeat expansion disease and the most common form of adult-onset muscular dystrophy [1-3]. The multisystemic symptoms are highly variable and involve different tissues and organs, including skeletal muscle, heart, brain, eyes, reproductive systems and the gastrointestinal tract. No cure to slow or stop the disease process is currently available.

The genetic basis of DM1 is an unstable (CTG)n expansion in the 3’ untranslated region of the DM protein kinase (DMPK) gene. Length of the (CTG)n segment is correlated with age of onset and severity of disease [1]. In the healthy population, the CTG repeat is polymorphic in length, with a triplet number that ranges from 5 to 37 (mostly below 15 [4]), whereas disease alleles may contain between 50 and >2000 triplets [1].

The DM1 pathogenic mechanism involves an RNA gain-of-function. Repeat-containing transcripts from the expanded allele accumulate in cell nuclei and alter the function of RNA-binding proteins, whose association with disease has been most intensively studied for muscleblind (MBNL) and CUG Binding Protein 1 (CUGBP1) [2]. In adult DM1 tissue, the resulting imbalance in availability of these antagonistic splicing regulators causes misregulation of events in which embryonic patterns of splicing occur. For example, myotonia and insulin resistance, two key features of DM1, may be caused by altered splicing of muscle chloride channel and insulin receptor transcripts, respectively [2]. Also effects on miRNA processing and function have been reported [5].

Results obtained with DM1 mouse models that inducibly express (CUG)n RNA show that features of RNA toxicity are reversible [6], strongly suggesting that reducing the level of expanded DMPK transcripts will be beneficial to patients. In the past decade, different antisense strategies have been developed with the intention to eliminate expanded DMPK transcripts via either antisense RNA expression, ribozyme activity, oligo-mediated RNase-H activity or RNA interference (RNAi) technology [7]. Reduction of DMPK transcript levels was achieved by several of these methods, but selectivity between toxic and normal-sized transcripts was generally low.

To be functional and safe for therapeutic use in DM1 patients, an antisense approach should ideally silence transcripts from the expanded allele only and leave normal-sized DMPK transcripts or unrelated transcripts containing (CUG)n segments intact. Since length of the (CUG)n repeat is the only consistent polymorphic difference between mutant and normal DMPK (pre)mRNAs, the repeat tract itself forms an attractive therapeutic target. Besides, despite the fact that normal and mutant DMPK transcripts are both made in the nucleus, (CUG)n-length selectivity may be enhanced by directing active compounds to this compartment where mutant transcripts accumulate after synthesis.

Earlier, we published on the identification and characterization of PS58, an antisense oligonucleotide (AON) complementary to the (CUG)n repeat [8]. PS58 is a (CAG)7 fully 2’-O-methyl (2’-OMe) phosphorothioate (PS) modified AON, which silences expanded (CUG)n transcripts in DM1 cell and animal models. Other in vivo AON studies directed at the expansion mutation made use of a (CAG)n morpholino to interfere with the (CUG)n repeat-MBNL interaction [9] or LNA (locked nucleic acid)/MOE (2’-O-methoxyethyl)-PS-type...
(CAG)n gapmers to recruit RNase H activity for degradation of (CUG)n-containing transcripts [10]. François et al. engineered a hU7-(CAG)15-snRNA which preferentially reduced pathogenic DMPK mRNA in DM1 cells, via a yet unknown mechanism [11].

Here, we report on specific properties that (CUG)n-directed AONs require for effective knockdown of expanded DMPK RNA. Triplet repeat AONs with different chemistries and lengths were tested in an unbiased screen for silencing capacity in a transgenic DM1 cell culture model expressing human DMPK (CUG)500 transcripts. AON length and nucleotide chemistry appeared to be major parameters for therapeutic efficacy. A selection of effective AONs was tested in DM1 patient myoblasts. Findings of this study are important for transition towards in vivo studies with AONs and ultimately for development of a specific AON-mediated therapy for DM1. As a first step in support of this transition we report on effects on in vitro complement activation by some of these AONs.

| RESULTS |

AONs complementary to DMPK’s (CUG)n repeat
A series of triplet repeat AONs that differed in length, chemistry and nucleotide composition was designed and analyzed for their ability to silence expanded DMPK transcripts in cultured cells (see AON description in Figure 1A). AONs were tested via transfection in the DM500 myoblast-myotube cell model [8]. The human DMPK (hDMPK) transgene in this model is under near-normal myogenic transcriptional control and produces hDMPK transcripts bearing a (CUG)500 repeat at levels comparable to that of endogenous mouse Dmpk (mDmpk) transcripts. It should be noted that mDmpk transcripts contain a CCG(CUG)₂(CAG)₂CUG sequence instead of the generally believed to be uninterrupted (CUG)n segment found in hDMPK transcripts. Monitoring AON silencing effects was done by Northern blotting to follow steady-state levels of full-length transgenic hDMPK transcripts and possible stable breakdown products. Analysis of endogenous mDmpk transcripts was used as a control. Real-time reverse-transcriptase PCR (RT-qPCR) was included for quantitative assessment of expression of defined transcript segments.

DNA-based (CAG)n AONs
DNA-type oligonucleotides are known to allow specific RNase-H mediated cleavage of RNA in a DNA-RNA heteroduplex [12]. For these AONs several chemical modifications have been developed that improve their metabolic stability, while maintaining hybridization affinity to target RNA and ability to recruit RNase H [13]. We tested three DNA-type (CAG)7 AONs in DM500 myotubes: PS56, a pure DNA AON; PS142, a PS-modified DNA AON; and PS260, a PS-modified chimeric AON, so-called gapmer, comprised of 2’-O-methyl (2’-OMe) wings on the 5’ and 3’ sides of a DNA gap. DNA AON PS56 did not show silencing activity (Figure 1A), probably because it was quickly degraded inside cells (Supplementary Figure S1). In contrast, PS142 and PS260 - both stabilized against nuclease breakdown - were able to silence expanded hDMPK mRNA for ~50% and ~80%, respectively (Figure 1A,B). Endogenous mDmpk mRNA was left intact. A concentration-response
Figure 1. Collection of triplet repeat AONs tested for their ability to silence expanded \( hDMPK \) mRNA in DM500 myotubes. (A) Summary of silencing efficiency. Endogenous \( mDmpk \) RNA was included as a negative control, since it lacks a pure (CUG)n repeat. AONs are grouped according to their main chemical modification: \( ^a \) DNA (capital); RNA (capital, underlined); PS, phosphorothioate linkage (**); 2'-OMe, 2'-O-methyl sugar modification (capital, underlined, bold); ENA, 2',4'-ethylene-bridged nucleic acid (capital, italics); PMO, morpholino phosphorodiimidade (lower case); \( ^b \) Cy3-labeled; \( ^c \) coupled to octaguanidine dendrimer. The means of at least three independent experiments for each AON are shown. Dashed line indicates 100% levels (mock samples). (B) Representative Northern blots with RNA isolated from AON-treated DM500 myotubes probed with a \( hDMPK \) and a \( Gapdh \) probe. Results of treatment with three AONs (200 nM) or mock treatment is shown. (C) Concentration-response curves of PS58 and PS142. DM500 myotubes were treated with a concentration series of 0.01-500 nM AON (see also Supplementary Figure S2). (D) RT-qPCR analysis of \( hDMPK \) RNA levels in DM500 myotubes after treatment with a selection of 2'-OMe AONs (200 nM, \( n \geq 3 \)). PCR amplicons were located in exon 15, either 5' or 3' to the (CUG)n tract.
analysis yielded an IC50 of 0.3 ± 0.4 mM for DNA/PS oligo PS142, which is about 1000-fold lower than that of 2’-OMe/PS lead compound PS58 (Figure 1C, Supplementary Figure S2 and [8]).

Oligo-length and base variations of 2’-O-methyl AONs
To investigate length as a potential parameter in the performance of PS58-type oligos, we tested a series of fully 2’-OMe-modified (CAG)n AONs consisting of four to thirteen triplets in the DM500 myotube culture system. All, except PS251 (CAG)4, effectively and selectively silenced (CUG)500 transcripts (>80% efficiency) (Figure 1A,B). A (CUG)7 2’-OMe sense oligo or 2’-OMe oligos directed against target sequences in the hDMPK transcript outside the repeat did not have a silencing effect (data not shown) [8]. The lack of activity of the (CAG)4 AON is best explained by assuming that a minimum oligo length is necessary for effective hybridization to the (CUG)n target needed for AON activity.

We wondered whether the nature of the nucleotide residue at the 5’ or 3’ end of the AON might be crucial for silencing and tested a repeat-shifted version, 2’-OMe-modified (AGC)7 (PS259). PS259 treatment resulted in a significant reduction of 85% of (CUG)500 transcripts without silencing mdmpk mRNA (Figure 1A).

Finally, a 2’-OMe-modified (CIG)7 AON was tested. The inosine residues should enable this AON, PS261, to hybridize to (CUG)n, (CAG)n, (CCG)n repeats and any combination of these triplets. Similar to PS58, PS261 induced 85% reduction of expanded mRNA (Figure 1A). Notably, endogenous mdmpk mRNA was also significantly reduced, albeit with only ~35% efficiency. This effect may be best explained by binding of (CIG)7 to the CCG(CUG)2(CAG)2CUG sequence in mdmpk mRNA. Besides, three low-affinity binding sites were identified that could have a cooperative, additive effect (11-13 nucleotides match; not present in hDMPK transcripts).

Silencing of expanded hDMPK transcripts by triplet repeat 2’-OMe AONs in DM500 myotubes was independently confirmed by RT-qPCR analysis (Figure 1D). The lower silencing efficiency measured by RT-qPCR (60-90%, versus 85-95% detected by Northern blotting) is attributed to technical differences in sensitivity intrinsic to the methods.

Morpholino phosphorodiamidate (CAG)n AONs
Morpholino phosphorodiamidate oligos are uncharged AONs widely used as blocking oligos for selective inhibition of gene expression [14]. A (CAG)8 morpholino (PS304) was tested for its ability to silence expanded hDMPK RNA in DM500 myotubes. The morpholino was coupled to an octaguanidine dendrimer (so-called Vivo-Porter) to facilitate cellular uptake. PS304 treatment caused a 70% reduction of (CUG)500 transcripts, leaving mdmpk mRNA intact (Figure 1A).

2’-O,4’-C-ethylene-bridged-nucleic-acid modified (CAG)n AONs
The bicyclic modification in 2’-O,4’-C-ethylene-bridged nucleic acid (ENA) confers strong nuclease resistance and high binding affinity to complementary single stranded RNA (average ΔT_m/modification = 5.5°C versus 1.3°C for 2’-OMe) [15] (Supplementary Figure S1).
We tested two fully ENA-modified AONs consisting of three and four CAG triplets, and three ENA/2'-OMe gapmers of five, seven and ten CAGs (PS139-135, Figure 1A). Only treatment with PS136 resulted in a small, yet significant reduction (30%) of hDMPK RNA expression.

The discrepancy in efficacy between ENA- and 2'-OMe-modified AONs may be caused by differences in subcellular targeting. To verify this possibility, we followed cellular uptake and routing of two fluorescently labeled AONs with these modifications. For these analyses we switched to the use of DM500 myoblasts, which display a rather flat morphology, rendering them more suitable for live cell imaging of AON localization than myotubes. Forty minutes after transfection, fluorescent PS58 oligo was present in the cell nucleus and signal intensity further increased up to 60 minutes (Supplementary Figure S3). This behavior was independent of the type of fluorophore used and fluorophore coupling did not affect PS58 silencing activity (data not shown). In contrast, labeled PS138, as a representative of the ENA AON collection, was concentrated in vesicle-like structures and rarely present in the nucleus itself. This pattern remained unchanged up to 48 hours after transfection (data not shown). We conclude that lower silencing efficiency of ENA-modified (CAG)n AONs observed in this study is due to their inability to reach muscle cell nuclei, where most expanded (CUG)n transcripts reside.

**Triplet repeat siRNAs**

Krol et al. [16] reported that (CXG)n triplet repeat hairpins in mRNAs are substrates for Dicer and produce short repeat RNAs that in turn may act as siRNAs against complementary repeats. We wanted to investigate whether in our cell model expanded hDMPK mRNAs could be silenced by introducing synthetic (CAG)n/(CUG)n siRNAs. Two different RNA duplexes were transfected in DM500 myotubes, PI-01 ((CAG)7/(GCU)7) and PI-02 ((GCA)7/(CUG)7) (Supplementary Table S1). An unrelated siRNA and PS58 were included as controls. RT-qPCR analysis demonstrated that none of the repeat siRNAs silenced hDMPK mRNA expression (Figure 2). From our AON stability assay, we conclude that it is unlikely that...
the inability to reduce expanded hDMPK transcripts is due to rapid intracellular degradation of the chemically unprotected siRNAs (Supplementary Figure S1).

**Mouse transcripts containing a small (CUG)n segment**

Transcripts that contain a (CUG)n segment, other than encoded by the hDMPK transgene, may also serve as targets for (CAG)n AONs in DM500 cells. Genes carrying a (CTG)n segment are rare, but at least nine transcripts in the mouse transcriptome contain a repeat of more than six triplets [8]. We investigated whether 2'-OMe/PS AONs of variable length and sequence ((CAG)5, (CAG)7, (CAG)10 and (CIG)7) acted on a subset of these transcripts expressed in DM500 myotubes. We analyzed transcript levels by RT-qPCR based on amplicons next to the repeat tracts (Figure 3; primer locations are depicted in more detail in Supplementary Figure S4). 

Ptbp1 (CUG)6, Txlnb (CUG)9 and Mapkap1 (CUG)26 - the longest (CUG)n tract in the mouse transcriptome - showed no significant changes in expression after AON treatment (Figure 3). Note that due to the location of the (CUG)9 tract in the very 5' end of the Txlnb gene, no suitable 5' amplicon could be designed to measure Txlnb transcripts.

We also wanted to analyze RNA expression via amplification across the (CUG)n triplet repeat. Semi-quantitative RT-PCR for Ptbp1 and Txlnb transcripts confirmed the RT-qPCR findings and showed no significant change in expression (Supplementary Figure S5A,B). Remarkably, quantification across the Mapkap1 (CUG)26 repeat did record significant silencing after AON treatment, especially by PS146 (CAG)10 (Supplementary Figure S5C). This effect was not seen, however, when segments flanking the (CUG)26 tract were amplified.
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in the same assay. Also Northern blotting, visualizing two types of Mapkap1 transcripts as a result of alternative polyadenylation [17] (Supplementary Figure S4), revealed only a minor reduction in RNA expression, while Western blotting revealed no significant changes in Mapkap1 protein expression (Supplementary Figure S5D,E). We therefore decided that PCR-based quantification should not rely on amplification across a medium-sized (CUG)n tract.

Given these observations, we wanted to exclude the possibility that traces of AONs that might still be present in purified RNA samples would interfere with RT-PCR analysis. Using a hybridization-ligation method, we determined that free AON concentration in our RNA preparations was less than 0.2 nM. To rule out interference of (CAG)n AONs with RT or PCR amplification, untreated DM500 RNA samples were spiked with 0.1-10 nM PS147, PS58 or PS146 and correspondingly used in RT-PCR analysis. Presence of AONs during RT-PCR amplification did not have an effect on Mapkap1 (CUG)26 levels nor on quantification of hDMPK (CUG)500 RNA (Supplementary Figure S6). In sum, we conclude that transcripts containing small (CUG)n tracts were largely unaffected by the (CAG)n and (CIG)n repeat oligos tested.

Mouse transcripts carrying a small (CAG)n segment

To examine the possibility that oligo-mediated hDMPK RNA breakdown would generate short (CUG)n RNAs that in turn might act as siRNAs on complementary (CAG)n transcripts (compare [16]), we measured levels of mouse transcripts with a small (CAG)n segment: Sec24b (CAG)15, Hcn1 (CAG)7+(CAG)20 and Nr3c1 (CAG)17. After treatment with PS147, PS58, PS146 and PS261 no significant change in expression of any of these transcripts was detectable by RT-qPCR analysis (Figure 4) ruling out an indirect effect of (CAG)n AONs on transcripts bearing a (CAG)n segment in the transcriptome.

(CUG)n length selectivity in DM1 patient myoblasts

A safe, antisense strategy for DM1 should differentiate between expanded and normal-sized hDMPK transcripts. To test this requirement, we used two DM1 myoblast cultures: one expressing hDMPK transcripts with 21 (normal) and 200 (mutant) triplets (abbreviated 21/200) and another with 5 and 1400 CUG triplets (abbreviated 5/1400) [18]. Activities of three 2’-OMe AONs, PS147, PS58 and PS146 were examined in these cultures, with use of Northern blotting to discriminate between normal and mutant hDMPK RNA. The three pure 2’-OMe/PS AONs behaved equally (Figure 5A): levels of (CUG)1400 and (CUG)200 transcripts were strongly decreased (65-95%). (CUG)21 transcripts appeared less susceptible to breakdown (45-55%) and as expected, (CUG)5 hDMPK mRNA was left intact. Silencing efficacy significantly correlated with the number of CUG triplets in the transcripts (Figure 5B).

Western blot analysis showed that silencing of hDMPK transcripts by PS146 (CAG)10 resulted in around 50% loss of DMPK protein after 72 h in 21/200 myoblasts (Figure 6). No effect of PS147 (CAG)5 or PS58 (CAG)7 treatment was observed at this time point.
Human transcripts containing a small (CUG)n segment

Also in the human transcriptome (CUG)n tracts are relatively rare [8,19]. Effects of a subset of 2'-OMe/PS AONs on transcripts bearing a (CUG)n repeat expressed in 5/1400 myoblasts were examined for two transcripts: BPGM (CUG)8 and MAP3K4 (CUG)10 by RT-qPCR. No reduction of BPGM transcripts was detected after treatment with PS147 and PS146, while only a small effect was seen with PS58 (25% reduction) (Figure 7A). Similar effects for BPGM (CUG)8 were detected in 21/200 myoblast cultures (data not shown).

Total MAP3K4 mRNA levels were not affected by 2'-OMe AON treatment (Figure 7B). A conspicuous observation was made with regard to an effect on MAP3K4 pre-mRNA processing. Analysis of the fate of alternative exon 17 [20], which contains the short (CUG)10 repeat that might serve as target, revealed that PS146 (CAG)10 was able to enhance exon 17 skipping (Supplementary Figure S7). Effects on alternative splicing by shorter (CAG)n oligos were only minor.

Investigating potential mechanisms of expanded hDMPK transcript silencing

We wondered whether exon skipping could also be the underlying or initiating mechanism of PS58-induced silencing of expanded hDMPK transcripts. Human DMPK pre-mRNAs are subject to extensive alternative splicing and events involving final exon 15, in which the (CUG)n tract is located, have been reported [21,22]. For analysis of fate of different
Figure 5. (CUG)n length selectivity in DM1 myoblasts. (A) Human myoblasts expressing either hDMPK (CUG)21 and (CUG)200 transcripts (21/200) or hDMPK (CUG)5 and (CUG)1400 transcripts (5/1400) were treated with a selection of CAG AONs. Northern blot analysis indicated that expanded hDMPK transcripts (‘exp’, arrow heads) were efficiently silenced by all AONs. (CUG)21 transcripts (‘normal’, double arrow heads) were moderately silenced. Only RNase-H recruiting PS260 substantially degraded (CUG)5 transcripts (‘normal’, double arrow head). One representative blot of three independent experiments is shown for each cell culture. Quantification of signals is summarized in the graphs at the bottom. (B) A significant correlation was demonstrated between the number of CTG triplets and silencing activity of PS147, PS58 and PS146 (P<0.05, Pearson’s correlation, r = -0.98), but not PS260.
Figure 6. Analysis of DMPK protein expression after AON treatment. Human myoblasts (21/200) expressing hDMPK (CUG)21 and (CUG)200 transcripts were treated with a selection of (CAG)n AONs. After 72 hours, protein lysates were made and DMPK protein (arrow head) levels were analyzed by Western blotting (n=3). Tubulin protein was used as a loading control. Lysate from Dmpk knock-out myoblasts served as reference (right two lanes).

Figure 7. Analysis of expression of two transcripts, each containing a small (CUG)n repeat, in DM1 patient myoblasts (5/1400) after transfection with a selection of AONs. RNA levels of (A) BPGM (CUG)8 and (B) MAP3K4 (CUG)10 were measured by RT-qPCR (n=3). Schemes illustrate exon-exon junctions and locations of PCR primers and amplicons. Dashed lines indicate 100% levels (mock samples).
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Figure 8. Human DMPK (CUG)500 RNA expression in DM500 myoblasts after transfection with PS58 or scrambled-PS58. Expression of four different transcript segments (little black bars) was analyzed by RT-qPCR (n=3): two in the 5' end of the transcript and two flanking the triplet repeat in the 3' end of the transcript. Scheme illustrates exon composition (numbered boxes) of hDMPK transcript, arrowhead indicates reported cryptic splicing site 3' to the triplet repeat [22]. Dashed lines indicate 100% levels (mock samples). No significant differences in response to the oligos were observed between the four segments.

hDMPK RNA segments in presence of AONs, we used a transcript scanning RT-qPCR approach for amplicons along the expanded hDMPK transcript in DM500 myotubes. Amplicons were designed in the 5' end of the gene (exon 1 - exon 2, exon 4 - exon 5) and on either side of the (CUG)n tract in exon 15 (Figure 8). No significant differences were found in signal strength for any of these hDMPK segments (70-85% reduction for all segments across the transcript, compared to mock-treated samples). This finding indicates that PS58-induced skipping of the (CUG)n tract during hDMPK pre-mRNA splicing is unlikely to be mechanistically involved.

Test-tube incubation of radioactively labeled, in vitro transcribed (CUG)90 RNA with a concentration series of PS58 (CAG)7 caused a mobility shift illustrating direct oligo-RNA association (Supplementary Figure S8). Similar shifts were seen after incubation of different (CAG)n and (CUG)n AONs with (CUG)90 or (CAG)90 RNAs (Supplementary Figure S8). To exclude that triplet-repeat 2'-OMe AONs had an intrinsic RNase activity, similar to ribozymes or DNAzymes [23], (CUG)n RNA was incubated with a series of different AONs in the presence of various concentrations of Mg²⁺, Mn²⁺ or Zn²⁺ (Supplementary Figure S8; data not shown). No (CUG)n breakdown products could be detected, indicating that under the conditions tested none of these AONs displayed RNase-like activity when complexed to (CUG)n RNA.

Using the same (CUG)90 RNA template, we could confirm that PS58, a fully modified 2'-OMe AON, was unable to induce RNase-H mediated degradation of expanded (CUG)n transcripts in vitro (Supplementary Figure S8). As positive controls, we used DNA-type AON PS142 and gapmer PS260, which both were able to recruit RNase-H to cleave (CUG)90 RNA.
Activity of gapmer PS260 was also examined in human myoblasts, but with an apparent lack of specificity (Figures 5 and 6). More than 60% of both expanded and normal-sized hDMPK transcripts was degraded. Similar lack of specificity was observed for BPGM (CUG)8 and MAP3K4 (CUG)10 RNAs, for which a 40% reduction in steady-state copy number was observed (Figure 7). Since the MAP3K4 exon17+ variant and the exon17- variant (which lacks the AON target site) were equally reduced by PS260, we conclude that RNase-H dependent degradation must have occurred in the nucleus, prior to exon 17 splicing (Supplementary Figure S7).

**Complement activation by triplet repeat AONs**

All triplet repeat oligos were well tolerated by cells in culture and essentially no toxic effects on growth or behavior were observed (data not shown). A well-known toxic side effect of antisense oligonucleotides in vivo, especially phosphorothioate-modified versions, is activation of the complement pathway [24]. As a prelude to later pre-clinical studies in animals and humans, we tested a selection of our PS-modified AONs with different chemistries and sequences for their ability to activate complement in plasma in vitro: full 2’-OMe (PS58, PS146, PS147, PS261), chimeric 2’-OMe/DNA (PS260) and pure DNA (PS142). A first generation oligonucleotide known to activate the complement pathway was included as a positive control [25]. Formation of complement split products C3a (marker for the classical and alternative pathway) and Bb (marker for the alternative complement pathway) was measured in plasma from cynomolgus monkeys and humans after addition of AONs (Supplementary Figure S9). In particular PS261 (CIG)7, and to a lesser extent PS146, PS147 and PS260, caused a significant dose-dependent increase in the concentration of Bb in cynomolgus plasma. Only PS261 and PS260 activated C3a formation in human plasma. Other AONs showed no significant or minor effects.

**DISCUSSION**

This study demonstrates that a range of triplet repeat AONs is capable of silencing expanded hDMPK transcripts. The long (CUG)n target that characterizes toxic hDMPK transcripts is a special RNA sequence in the sense that it can form a stem-loop structure that folds almost like an antiparallel double-stranded helix, despite the periodic U•U mismatches [26]. We conclude that the thermodynamically stable hairpin structure, known to sequester members of the MBNL protein family, does not hamper accessibility of antisense repeat AONs, when in the cellular context. The (CUG)n repeat, whose length differs between normal-short and mutant-expanded hDMPK transcripts, is therefore an attractive therapeutic target.

Chemical modifications of the sugar phosphate backbone of AONs serve to confer resistance against nucleases to extend cellular half-life [13]. The silencing activity of modified AONs PS142 and PS260 versus the inability of pure DNA AON PS56 to induce breakdown clearly illustrates the relationship between stability and efficacy. Oligo chemistry may also determine nucleocytoplasmic distribution of AONs. As target transcripts in DM1 are concentrated in the nucleus, an AON must be capable to enter this compartment to
become useful. Microscopic evidence is provided that ENA-modified AONs remain in vesicular structures and only inefficiently reach the nucleus, rendering them a poor choice for repeat-directed antisense DM1 strategies. We have no simple explanation for this finding, as similarly modified 2’-OMe RNA/ENA chimeras have shown activity in the nucleus during exon skipping for Duchenne muscular dystrophy [27]. Effects of nucleotide sequence on subcellular oligo localization cannot be excluded at this point. Also the type of cell model and transfection method used may have an effect.

Not all types of AONs were equally efficient and specific, but as we compared only triplet repeat oligos here, some conspicuous observations are worth mentioning. First, as an explanation for the failure of (CUG)n/ (CAG)n triplet repeat siRNAs to induce an RNAi response, we assume that a combination of chemistry and oligo sequence must be responsible. The poor performance of the two duplexes tested can be the result of unfavorable subcellular distribution of siRNAs, although the RNAi machinery is known to be active in the nucleus [28]. Alternatively, the (CUG)n RNA hairpin may be insufficiently accessible to certain components of the RNAi pathway in our myogenic cell model. Very recently, a comparable triplet repeat siRNA was successfully tested in vivo in skeletal muscle in a mouse model for DM1. (CUG)220 expanded RNA and also Txlnb (CUG)9 transcripts were reduced, but not Mapkap1 (CUG)25 RNA [29].

Secondly, oligo PS261 (CIG)7 was initially considered a promising new lead compound, because of its predicted ability to bind to (CUG)n as well as (CAG)n transcripts, with potential for treatment of DM1 and polyQ diseases like Huntington’s disease and several spinocerebellar ataxias. From the complement activation assay we learned, however, that this AON activated the complement cascade, rendering it a less favorable candidate for further development. Virtually none of the other AONs showed major adverse effects in vitro, with the best performing oligo being PS58. Screening for toxicity profiles in animals in vivo will be a logical next step.

Thirdly, RNase-H recruiting AON PS260 appeared to have prominent silencing capacity, but also suffered of lack of target specificity. Further characteristics of these DNA-type AONs will be discussed below in the context of mechanistic considerations.

The main focus of our study had to do with AONs based on 2’-OMe/PS chemistry. For these AONs we demonstrated that oligo length may provide a means to introduce selectivity between expanded transcripts from a mutated hDMPK allele and RNA containing a normal, short (CUG)n tract. The shortest variant in our series of four to thirteen CAG triplets was unable to knockdown expanded transcripts in DM500 myotubes. The hybridization strength of the four CAG triplet AON may have been insufficient for a stable interaction with a (CUG)n segment in the living cell, even though the theoretical $T_m$ for this interaction is around 39°C. AONs longer than four triplets all very efficiently silenced transgenic (CUG)500 transcripts. Significant (CUG)n-repeat length dependent activity in patient cells was displayed by (CAG)5, (CAG)7 and (CAG)10 2’-OMe/PS AONs. Between them they showed no significant difference in activity towards normal-sized hDMPK transcripts: (CUG)5 transcripts, expressed by ~40% of the Caucasian population [4], were left intact and (CUG)21 transcripts were ~50% reduced by all three AONs. In contrast, differential effects were observed at the DMPK protein level,
which might indicate a correlation with translation efficiency of remaining hDMPK transcripts. It should be noted that we cannot predict at the moment whether partial reduction of normal-sized transcripts will have any adverse consequences. We know that Dmpk knock-out mice have relatively mild phenotypes [30], hence a low level of remaining Dmpk protein might be sufficient to protect functional and structural integrity of cells in which it is expressed. Another still unresolved issue is the minimum efficacy needed for a beneficial effect. Partial suppression of toxic (CUG)n RNA may already be of clinical relevance in DM1 patients.

Expression of genes with a small (CTG)n repeat in myogenic cells was essentially unchanged upon treatment with 2'-OMe/PS AONs, except for MAP3K4. PS146 (CAG)10 enhanced skipping of MAP3K4 exon 17 in which the (CUG)10 repeat is located. This (CUG)10 repeat is unusual as it is located in an alternative exon, where it might function as an exon splicing enhancer [31]. To what extent exon 17+ and exon 17- transcript variants contribute to protein production is unknown. More importantly, it is unclear whether a shift in MAP3K4 isoform ratio would have a biological effect. Nothing is known about functional differentiation generated by inclusion of the 50 amino-acid stretch encoded by in-frame exon 17 (including 10 alanines encoded by the repeat tract). The minor shift in MAP3K4 variant ratio induced by smaller (CAG)n AONs is essentially within the normal variation observed in mock samples. Note that since splicing is a nuclear process, we can conclude that PS146 (CAG)10 must have been active inside the nucleus.

It is difficult to reconcile all observations regarding Mapkap1 (CUG)26 transcripts. First, as also seen with MAP3K4 transcripts, oligo length is an important parameter for AON activity and side effects seem to intensify with increased oligo length. Secondly, we provide ample evidence that our oligos did not interfere with RT-PCR quantification. Third, we propose that special care should be taken with RNA quantification based on amplicons covering a triplet repeat of more than ten units. These amplicons will never meet requirements for RT-qPCR analysis, but they may also be useless in semi-quantitative RT-PCR. For that reason, we generally used segments outside the repeat region for PCR quantification. Similar warnings regarding RT-PCR and use of AONs have been issued by others [32]. Given the location of the (CTG)26 repeat in between two alternative polyadenylation sites of the Mapkap1 gene [17], we cannot exclude length-dependent AON effects on splicing, transcription termination or polyadenylation. Clearly, more work is needed to be able to unequivocally interpret these data.

What do we know about the molecular mechanism underlying silencing activity of hDMPK transcripts by triplet repeat AONs examined here? Although the goal of this study was not to identify distinct pathways or describe novel interference principles, our findings provide important new insight in potential activities of triplet repeat AONs.

In a series of simple biochemical test tube experiments, we first investigated whether triplet repeat 2'-OMe/PS AONs might carry intrinsic endoribonuclease activity, similar to ribozymes or DNAzymes [23]. In none of the experiments RNase activity was detected. Although we also tested several cell-free or protein-free conditions (data not shown), it cannot be excluded that the right cellular reaction conditions were not fully reproduced.
We provide evidence that PS260, a (CAG)7 gapmer, efficiently reached various (CUG)n RNA targets, irrespective of repeat length, and recruited endogenous RNase H for a first endonucleolytic cleavage. We expect that unprotected 5’ and 3’ RNA segments will be degraded after initial cleavage by conventional exonucleases as part of the quality control system in the cell [33]. Surprisingly, PS260 showed only little selectivity between expanded and normal-sized hDMPK transcripts in patient cells, suggesting that either RNase H activity took place in both the nucleus and the cytosol, or that degradation already occurred swiftly, during or shortly after RNA synthesis in the nucleus. Our observations contrast with a report that an LNA-based repeat gapmer did cleave hDMPK (CUG)40, but not (CUG)12 transcripts [10]; and also with a study in which a (CAG)6 LNA gapmer did not reduce CASK (CUG)16 RNA [34]. The reason for these discrepancies should probably be found in oligo chemistry, concentration or model system used.

Nucleotide analogs like morpholinos or modifications like 2′-OMe do not support RNase-H dependent degradation. These AONs must therefore use distinct pathways, explaining why PS58, a 2′-OMe/PS (CAG)7 AON, and PS260, displayed differential (CUG)n-length dependent silencing.

The RNAi pathway is unlikely to be involved in activity of fully 2′-OMe/PS AONs or (CAG)n morpholinos. Imperfect hybrids of 2′-OMe/PS or morpholino (CAG)n AONs, or perfect hybrids that these AONs may form with (CUG)n transcripts, will not be recognized by the RNAi machinery [35-37]. Furthermore, the size of the shortest effective AON (15 nucleotides) and tolerated 5′ modifications like a fluorescent group (as in FAM-PS58) or a peptide (data not shown) render involvement of the RNAi pathway highly unlikely [38,39]. Also, we did not find evidence for AON-induced processing of expanded hDMPK transcripts into (CUG)n siRNAs that could trigger degradation of (CAG)n transcripts.

Fully 2′-OMe/PS-modified AONs, like PS58, and morpholinos are usually designed and employed to serve as blocking oligos to modulate splicing or prevent translation [13,40]. As discussed above, PS146, a 30 nt-long 2′-OMe/PS AON, exhibits splicing modulating activity. We could not detect a similar activity of (CAG)n AONs towards hDMPK (CUG)500 transcripts. The most logical explanation for this observation is the location of the (CUG)n repeat in the final exon of the hDMPK gene. The only reported splice acceptor, located around 35 nt 3′ of the (CUG)n tract, is only rarely used [22,41] and probably too weak to be amenable to manipulation.

A (AGC)8A morpholino reportedly blocked Mbnl1 protein binding to transgenic (CUG)250 transcripts in a mouse model for DM1-related RNA toxicity [9]. In that study, cytoplasmic levels of expanded transcripts raised upon treatment, but the total amount of (CUG)250 transcripts was ~50% reduced. A comparable reduction was reported for expanded Huntingtin (CAG)n RNA using a (GCT)6G LNA-type blocking oligonucleotide [42]. The molecular mechanism underlying activity of the AGC morpholino is unknown, but it was suggested that the displacement of expanded transcripts to the cytoplasm may have induced hDMPK mRNA decay. We propose that a similar mechanism may be at play for our (CAG)n morpholino and the 2′-OMe/PS AONs examined here. Alternatively, this class of AONs may activate a yet to be identified RNase or RNA decay program in nucleus or cytoplasm.
Our current working hypotheses are that the interaction between (CAG)n AONs and the (CUG)n repeat attracts proteins involved in RNA degradation or that AON binding displaces RNA protecting proteins resulting in naked RNA, which is extremely sensitive to RNases. Further detailed studies into these interesting possibilities are warranted.

In sum, we provide further evidence that the expanded (CUG)n segment in hDMPK mRNA, being the product from the only known mutation in DM1, forms an attractive target for antisense oligonucleotide therapy. For development of future treatment modalities, chemical structure and oligo length in combination with the rather special sequence content needed – i.e., the complementary (CAG)n repeat character – have now been identified as crucial determinants. By extrapolation of findings, our results also open up interesting possibilities for therapy of other triplet repeat diseases.

| MATERIALS AND METHODS |

Cell culture
Immortalized DM500 myoblasts were derived from DM300-328 mice [43] and cultured and differentiated to myotubes as described [8]. Human DM1 myoblast lines were derived and cultured as described [18].

Oligonucleotides
AONs and siRNAs were complementary to the (CUG)n repeat. Their sequence and chemistry are listed in Figure 1A and Supplementary Table S1, except for control oligos scrambled-PS58: 5'-CAGAGGACCACCAGACCAAGG-3'; and PS57: 5'-CUGCUGCUGCUGCUGCUGCUG-3' (both fully 2'-OMe/PS-modified). All AONs and siRNAs were provided by Prosensa (Leiden, NL), except morpholino PS304, which was purchased coupled to an octaguanidine dendrimer from Gene Tools (Philomath, OR, USA) and control siRNA was purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA).

Oligo transfection
AONs were transfected using polyethyleneimine (PEI; ExGen 500, Fermentas, Glen Burnie, MD, USA) according to manufacturer’s instructions. Typically, 5 μl PEI/μg AON was added at a final AON concentration of 200 nM in differentiation medium to myotubes on day five of myogenesis or in Opti-MEM (Invitrogen, Carlsbad, CA, USA) to myoblasts. For concentration-response experiments, oligo concentrations between 0.01 nM and 500 nM were applied. Four hours later fresh medium was supplemented to a maximum volume of 2 ml medium/well. Medium was changed 20 hours later. RNA was isolated 48 hours after start of transfection. PS304 was tested following the same protocol with the exception that no transfection reagent was used.

SiRNAs were administered twice (on subsequent days: day four and five of myogenesis) using a Lipofectamine 2000-based transfection protocol (Invitrogen) according to manufacturer’s instructions, at a final concentration of 50 nM. Four hours following addition of the transfection mix and 24 hours after start of the second transfection medium was changed. RNA was isolated 48 hours after the second transfection.
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**Oligo stability**

AONs (7.5 pmoles) were radioactively labeled by T4 polynucleotide kinase in the presence of [γ-32P]-ATP and then purified on G50 columns followed by ethanol precipitation.

DM500 myoblast whole cell extract was prepared by scraping approx. 1x10^6 cells per well in PBS containing 1 mM EDTA. Cells were collected via centrifugation (1000 rpm, 5 min), packed cell volume (PCV) was estimated and cells were resuspended in 1x PCV of 10 mM Tris-HCl pH 8.0, 1.5 mM MgCl\(_2\), 10 mM KCl, 1 mM DTT and 1x Protease Inhibitory Cocktail (Roche, Almere, NL). Cell suspensions were placed on ice for 15 min and homogenized using a pestle. Whole cell lysate was collected after centrifugation (14,000g, 10 min).

AONs (approx. 2 pmoles) were incubated with 5 µl whole cell lysate at 37°C for 0, 3 or 24 hours, reaction was stopped by adding 140 mM sodium acetate, pH 5.0, phenol/CHCl\(_3\) and water phase was collected (14,000g, 10 min, 4°C). Oligo was precipitated by the addition of three volumes of ethanol. The oligo precipitate was washed with 70% (v/v) ethanol, air dried and dissolved in sample buffer (75% deionized formamide, 25% glycerol). Samples were loaded on a 12% (w/v) acrylamide/ bisacrylamide (37.5:1) denaturing-urea (8 M) PAGE gel in 1x MOPS buffer. After running, gels were dried at 70°C for 2 hours and exposed to X-ray film (X-Omat AR, Kodak, Rochester, USA).

**RNA isolation**

RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer’s protocol.

**Northern blotting**

Northern blotting was done as described [8]. Random-primed 32P-labelled human DMPK (2.6 kb cDNA), rat Gapdh (1.1 kb cDNA) and mouse Mapkap1 (1.1 kb cDNA) probes were used. Signals were quantified by phospho-imager analysis (GS-505 or Molecular Imager FX, Bio-Rad) and analyzed with Quantity One (Bio-Rad) or ImageJ software. Gapdh levels were used for normalization. RNA levels of control samples were set at 100%.

**RT-PCR**

Primer sets for PCR were designed using Primer-BLAST [44] in the NCBI database and validated in silico using OligoAnalyzer 3.1 [45] to prevent formation of hairpins and dimers during amplification. Resulting products were visualized on agarose gels and sequenced to verify identity and triplet repeat length. Primer sequences are listed in Supplementary Table S2.

For reverse transcription (RT), typically 0.5 µg RNA was subjected to cDNA synthesis using the SuperScript first-strand synthesis system with random hexamer primers in a total volume of 20 µl. For RT-PCR experiments in presence of AONs, AONs were added to the RNA to reach the indicated final concentration and incubated for 15 min on ice before starting the RT reaction.

For semi-quantitative RT-PCR, one µl of cDNA preparation was used in a PCR according to standard procedures. In RT- control experiments, reverse transcriptase was omitted.
The signal for β-actin was used for normalization. Cycle number was 18 for β-actin and 22-31 for other primer sets. PCR products were analyzed on 1.5-2.5% agarose gels stained by ethidium bromide. Quantification of signals was done using Labworks 4.0 software (UVP BiolImaging Systems, Cambridge, UK). Values for control samples were set at 100%.

For real-time RT-PCR (RT-qPCR), all procedures were performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [46]. Amplification efficiency of primer sets was determined using standard curves (serial dilutions; 10-5000 fold range). Primers sets displaying less than 90% efficiency in product amplification were ruled out and re-designed. cDNA preparations (see RT reaction above) were diluted 200-500 fold to avoid PCR inhibition by RT components. 3 µl of this dilution was mixed in a final volume of 10 µl containing 5 µl 2X Sybr Green mix (Roche Applied Science, Almere, NL) and 400 nM of each primer using the CAS-1200 automated pipetting system (Corbett Life Science/Qiagen, Venlo, NL). No template control (NTC) and no reverse transcriptase control (RT-) were included in each qPCR run to detect possible contaminations. Samples were analyzed using the Rotor-Gene 6000 (Corbett Life Science) or CFX96™ Real-time System (Bio-Rad). A melting curve was obtained for each sample in order to confirm single product amplification. Relative mRNA levels were calculated using the ΔΔCt method [47]. Both Actb and Gapdh were used for normalization. We validated that these reference genes were not affected by the treatment and had an optimal M stability value [48] under all experimental conditions. Values of control samples were set at 100%.

AON hybridization ligation assay
PS58 concentrations were determined by a hybridization-ligation assay as published by Yu et al. [49] with a few modifications: Template DNA probe (5'-GAATAGACGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG-biotin-3') and ligation DNA probe (5'-P-CGTCTATTC-digoxigenin (DIG)-3') were used. Samples were incubated with the template probe (50 nM) at 37°C for 1 hour and hybridized samples were transferred to streptavidin-coated plates. Subsequently, plates were washed four times and the DIG-labeled ligation probe (2 nM) was added and incubated for 30 min at ambient temperature. DIG label was detected using an anti-DIG-POD (1:7,500; Roche Diagnostics), visualized with a 3,3',5,5'-tetramethylbenzidine substrate (Sigma Aldrich, Zwijndrecht, the Netherlands). The reaction was stopped using maleic acid (345 mM, Sigma Aldrich). Absorption was measured at 450 nm using a BioTek Synergy HT plate reader (Beun de Ronde, Abcoude, NL). All samples and calibration curves were diluted (fit to criteria of the assay) in PBS. Absorption was read against a calibration curve of PS58 in PBS. A sample containing only PBS was used as correction for background.

Live cell imaging
DM500 myoblasts were cultured on gelatin-coated glass-bottom Willco dishes (Willco Wells, Amsterdam, NL). Cells were analyzed directly after addition of the transfection mix containing FAM-PS58 or Cy3-PS138 in RPMI medium without phenol red and including 10 mM HEPES and 10% FCS, (Invitrogen). A Zeiss LSM510meta confocal laser-scanning microscope was used, equipped with a temperature controlled CO₂ incubator (type S) and sample stage. For
recording of oligo uptake, we used a PlanApochromatic 63x, 1.4 NA oil immersion DIC lens (Carl Zeiss GmbH, Jena, Germany).

### Western Blotting

DM1 patient 21/200 myoblasts or DM500 mouse myotubes were transfected with PS147, PS58, PS146, PS260 or PS261 as indicated (200 nM) or mock transfected and 72 h later lysed in lysis buffer (TBS, 1% Nonidet P-40, 1 mM PMSF and Protease Inhibitory Mixture (Roche, Almere, The Netherlands)) on ice. Cleared lysates were separated on an 8% SDS/PAGE gel and transferred to PVDF (Amersham Pharmacia Biotech). Blots were incubated with DMPK-specific antibody B79 [21] or anti-SIN1/Mapkap1 antibody (ab71152, Abcam, Cambridge, UK) and β-tubulin monoclonal antibody E7 (Developmental Studies Hybridoma Bank, University of Iowa), followed by ECL detection. Quantification of signals was done using ImageJ software. Values for mock-treated samples were set at 100%.

### Experiments using in vitro transcribed triplet repeat RNA

(CUG)n and (CAG)n RNA was generated by in vitro transcription of a hDMPK (CTG)90 DNA template carrying flanking T7 and SP6 promoters. The hDMPK (CTG)90 template was generated by PCR using a hDMPK cDNA construct bearing a (CTG)90 repeat [50], T7-hDMPK forward primer 5’-GAATTTAATACGACTCACTATAGGGAGAACGGGGCTCGAAGGGT-3’ and SP6-hDMPK reverse primer 5’-ATTTAGGTGACACTATAGAAGGGCGTCATGCAACAGAAAA-3’ and sequenced to verify triplet repeat number. (CUG)90 and (CAG)90 RNAs were synthesized in presence of [α-32P]GTP, using the MEGAscript® in vitro transcription kit (Applied Biosystems/Ambion, Austin, TX, USA) according to manufacturer’s protocol.

To confirm AON binding to expanded triplet repeat RNA, (CUG)90 RNA was incubated with a concentration series (1, 10 and 100 μM) of PS58 in 50 mM Tris-HCl, pH 7.5 and 25 mM MgCl₂ for 2 hours at 37°C. Sample buffer was added to a final concentration of 50% deionized formamide and 30% glycerol and samples were run at room temperature on a denaturing polyacrylamide gel (5% acryl/bisacryl, 8 M urea) in 1x MOPS. Gels were dried and exposed to X-ray film (X-Omat AR, Kodak).

To reveal binding specificity, (CUG)90 and (CAG)90 RNAs were incubated with 100 μM AONs in the presence of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 25 mM MgCl₂ and 1 mM EDTA for 2 hours at 37°C. Samples were next analyzed by electrophoresis as described above.

To examine RNase-H dependent activity, (CUG)n RNA was dissolved in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂ and 1 mM DTT and incubated with 60-100 μM AON in the presence of 20 U/ml RNase H for 1 hour at 37°C. Samples were analyzed by electrophoresis as described above with the exception that the 1x MOPS running buffer was heated to 70°C before start of electrophoresis to induce dissociation between AONs and (CUG)n RNA.

To examine intrinsic oligo catalytic activity, (CUG)n RNA was dissolved in 100 mM Tris-HCl, pH 7.5, 100 mM NaCl and 1 mM EDTA and incubated with 100 μM AON for 2 hours at 37°C in the presence of 3.5 or 25 mM MgCl₂. Samples were analyzed by electrophoresis as described above.
Complement activation assay
The potency of AONs to activate the complement pathway was demonstrated using Li+-heparin plasma from cynomolgus monkeys (kindly provided by CIT, France) and Li+-heparin plasma from healthy human donors (Sanquin, The Netherlands). AONs were added to plasma in various concentrations (25, 50, 150 and 300 µg/ml in a dilution of approx. 1:20 (volume AON/volume plasma)) and the samples were incubated at 37°C for 30 min. The reaction was terminated by putting the samples on ice and adding ice-cold diluents according to the manufacturer’s protocol (Quidel, San Diego, CA, USA). Bb and C3a concentrations were determined by ELISA (Quidel). First generation oligonucleotide PS455 (ISIS 5132) was included as a positive control, as this oligo has been shown to activate the complement pathway [25].

Statistical Analysis
Northern blot and PCR signals were analyzed using an unpaired Student’s t-test or a one-way ANOVA. All values in graphs are presented as the mean ± SEM. Pearson’s correlation was applied to evaluate whether a correlation exists between silencing efficacy and number of CTG triplets in hDMPK alleles in human myoblasts. Differences between groups were considered significant when P<0.05: *, P<0.01: **, P<0.001. Statistical analyses and sigmoidal concentration curve fitting were performed with GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA, USA).

| ACKNOWLEDGMENTS |
This work was supported by grants from SenterNovem (a Dutch agency in the Ministry of Economic Affairs), the Prinses Beatrix Spierfonds (in particular the Stichting Spieren voor Spierziekten [War07-34]), the Association Française contre les Myopathies and the Muscular Dystrophy Association - USA. We thank members of the Department of Cell Biology for discussions and continued support and Sjef de Kimpe and Gerard Platenburg (Prosensa Therapeutics, Leiden, The Netherlands) for help during the initial period of this project. We are grateful to Geneviève Gourdon (Hôpital Necker-Enfants Malades, Paris, France) for kindly providing mice from the DM300-328 line to establish the immorto DM500 cell model and to Denis Furling (Institut de Myologie, Paris, France) for providing human DM1 myoblast lines. We thank Makoto Koizumi (Sankyo Co., Ltd., Tokyo, Japan) for providing ENA nucleosides as building blocks for the synthesis of ENA-modified oligonucleotides. A.G.-B., S.A.M.M., J.v.d.G., S.B. and J.C.T.v.D. report being employed by or having an equity interest in Prosensa Therapeutics B.V. Data described in this paper are the subject of a patent application (inventors D.G.W. et al.).
REFERENCES


Figure S1. AON stability in cell extract. AONs were end-labeled with [γ-32P] ATP, incubated in DM500 whole cell extract for 0, 3 or 24 hours and then analyzed by polyacrylamide gel electrophoresis. PS56 was almost completely degraded after 24 hours. Chemically modified AONs PS58 and PS136, and siRNA PI-02 were still present after 24 hours incubation. Numerals at the bottom indicate quantification by phosphoimager analysis.

Figure S2. Concentration-response curve of PS142 including raw data. DM500 myotubes were treated with a concentration series of 0.01-500 nM of this AON. RNA was isolated 48 hours post-transfection and hDMPK transcript levels were analyzed by Northern blotting. Data points are the mean of at least three measurements.
Figure S3. Differential uptake and localization of AONs in DM500 myoblasts. DM500 myoblasts were transfected with (a-f) FAM-PS58 or (g-l) Cy3-PS138. Uptake was visualized by live cell imaging. Time points indicate minutes after addition of the oligo-transfection reagent complex to the culture medium. Phase contrast (e, k) and dashed lines help to recognize cell boundaries (see also merge). Essentially no nuclear localization was detected for ENA-modified PS138, whereas 2’-OMe/PS-modified PS58 showed prominent nuclear localization. Intense signals in panels a-f represent FAM-PS58-polyethyleneimine complexes in the medium. Representative images of two independent experiments are shown. The type of fluorophore used did not affect AON behaviour (data not shown). Bars: 20 µm.
Figure S4. Schematic representation of triplet repeat transcripts and segments thereof, analyzed by RT-(q)PCR. Boxes indicate exons. (CUG)n and (CAG)n tracts are highlighted in red and blue, respectively. PCR primers are represented by arrows. Transcript lengths are based on NCBI Reference Sequences and indicate nucleotides. pA indicates alternative polyadenylation site in Mapkap1.
Figure S5. Expression of mouse transcripts, carrying a small (CUG)n repeat, after transfecting DM500 myotubes with a selection of AONs: (A) Ptbp1 (CUG)6, (B) Txlnb (CUG)9 and (C) Mapkap1 (CUG)26. 
Semi-quantitative RT-PCR was used to be able to amplify a segment across the repeat. Schemes on top illustrate exon-exon junctions, locations of PCR primers and amplicons. Dashed lines indicate 100% levels (mock samples). (D) Northern blot containing RNA isolated from DM500 myotubes treated with repeat AONs or mock-treated. The blot was incubated with a Mapkap1 probe and a Gapdh probe for normalization. Two Mapkap1 transcripts were detected which result from alternative polyadenylation (Schroder et al. 2004 Gene, 339, 17-23). The (CUG)26 tract is only present in the long form (Supplemental Figure S4). Only a minor reduction in the level of both transcripts was observed upon AON treatment (indicated at the bottom). (E) Mapkap1 protein levels were measured by Western blotting 72 h after mock or AON treatment (n=3). No significant changes in expression were observed.
Figure S6. Control experiments to exclude perturbing effects of AONs during RT-PCR quantification. (A) Purified RNA from DM500 myotubes was incubated with PS147, PS58 or PS146 (0 - 10 nM) and subsequently used in a semi-quantitative RT-PCR assay based on an amplicon containing the (CUG)26 tract to measure Mapkap1 (CUG)26 levels. Actb RNA was used for normalization (see numerals at the bottom). None of the three AONs influenced RT-PCR efficiency, not even at high concentration. (B) The same RNA samples were used in a RT-qPCR assay to measure hDMPK (CUG)500 RNA levels. Amplicons were located in exon 15, 5’ and 3’ of the (CUG)500 tract. Again, no effect of (CAG)n AON pre-incubation on RT-PCR efficiency was observed. Dashed lines indicate 100% level (mock samples).
Figure S7. (CAG)n oligos can modulate alternative splicing of the (CUG)n-containing exon in the MAP3K4 primary transcript. (A) DM1 patient 5/1400 myoblasts were transfected with a selection of AONs followed by semi-quantitative RT-PCR amplifying a segment containing MAP3K4 exon 17, including the (CUG)10 repeat. The ex17- variant increased upon PS146 (CAG)10 treatment, while the level of the ex17+ variant decreased. Gapmer PS260 (CAG)7 reduced expression of both MAP3K4 variants. Quantification of results obtained with all four AONs are shown in B (ex17- fraction) and C (total MAP3K4 RNA levels). Dashed line indicates 100% level (mock sample).
Figure S8. Test-tube experiments using *in vitro* synthesized RNA bearing a (CUG)90 or (CAG)90 tract. (A) Schematic representation of the hDMPK gene segment flanked by T7 and SP6 promoters, obtained by PCR, used for the synthesis of RNAs with a (CUG)90 or (CAG)90 tract. (B) (CUG)90 RNA was incubated for 2 hours at 37°C with different concentrations of PS58 (CAG)7 and then loaded on a polyacrylamide gel. A concentration-dependent mobility shift was observed revealing oligo-RNA binding. (C) The same approach was followed with different (CAG)n or (CUG)n AONs and with (CUG)90 RNA and (CAG)90 RNA, with comparable results. (D) Incubation of (CUG)90 RNA with 2'-OMe/PS (CAG)n AONs in presence of 3.5 or 25 mM Mg²⁺ for 2 hours at 37°C did not show any proof of ribozyme activity. (E) (CUG)90 RNA was incubated with PS58 (2'-OMe/PS), PS260 (2'-OMe/DNA/PS) or PS142 (DNA/PS) in presence of RNase H for 1 hour at 37°C. RNase H only degraded RNA in a RNA-DNA duplex (stable products indicated by arrowheads). Chemistry and sequence of AONs is listed in Figure 1A. W=position of the wells; F= front.
Figure S9. Complement activation assay. Complement activation by a selection of AONs was examined in plasma from (A) cynomolgus monkeys or (B) humans. Formation of split products Bb (left panels) and C3a (right panels) was determined by ELISA. See Figure 1A for AON sequence and chemistry. ISIS 5132 was used as a positive control (Rudin et al. (2001) Clin. Cancer Res., 7, 1214-1220).

Table S1. SiRNAs (RNA duplexes) used in this study.

| PI-01:                      | 5’-CAGCAGCAGCAGCAGCAGCAG-3’ + 5’-GCUGCUGCUGCUGCUGCU-3’ |
|                            |                                                       |
| PI-02:                      | 5’-GCAGCAGCAGCAGCAGCAGCAGCAG-3’ + 5’-CUGCUGCUGCUGCUGCU-3’ |
| Control:                    | 5’-CAAGAACGAAUUGCUAGACATT-3’ + 5’-UGUCAGCAAUCGUUCUUGTT-3’ |

Capital: DNA. Capital, underlined: RNA.
Table S2. PCR primers used in this study (5' -> 3'):

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<th>Reverse Primer</th>
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<td>mActb Rv</td>
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<td>hDMPK e15(3’) Fw</td>
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<tr>
<td>hDMPK e15(3’) Rv</td>
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<td>hBPGM (3’) Rv</td>
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Primers for *mGapdh* were described earlier (Fu et al. (2007) J. Biomed. Mater. Res. A, 83, 770-778). **Semi-quantitative PCR:** Primers for *Ptbp1, Txlnb* and *Mapkap1* were described earlier (Mulders et al. (2009) Proc. Natl. Acad. Sci. USA, 106, 13915-13920).
APPENDIX
INTRODUCTION
The data included in this appendix are aimed at confirming experimentally whether PS58 employs the RNA interference (RNAi) pathway to silence expanded DMPK transcripts. We tested this AON in DM1 patient myoblasts where levels of Argonaute2 (AGO2) were reduced by means of siRNAs. The AGO2 protein is required by the RNA-induced silencing complex (RISC) to perform the endonucleolytic cleavage of target mRNA in the RNAi pathway [1]. We expect that, if PS58 works via this pathway, reduced levels of AGO2 protein will decrease the silencing activity towards expanded DMPK transcripts.

MATERIAL AND METHODS
Cell culture
Primary human myoblasts from a DM1 patient expressing DMPK transcripts with 13 (healthy allele) and 800 (expanded allele) CUG triplets were cultured on gelatin-coated plates. The proliferation medium used consisted of Ham F10 medium with GlutaMAX™ and 20% HyClone grow serum.

siRNA nucleofection
For AGO2 knock-down experiments, SMART-pool siRNA targeting human AGO2 (previously named EIF2C2) and control siRNA targeting mouse Mypt1 (Ppp1r12a) were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). The target sequences of AGO2 siRNAs are (5’>3’) CAAGCAGGCCUUCGCACUA, GGUCUAAAGGUGGAGAUAA, CCAAGGCGGUCCAGGUUCA and GCACGACUGGGACACGAA. The target sequence for control siRNA is (5’>3’) UGUCAGCAAAUCGUUCUUG. SiRNA transfection was performed using Amaxa™ P5 Primary Cell 4D-Nucleofector™ X Kit L (Lonza, Basel, Switzerland) following manufacturer’s instructions: human myoblasts were trypsinized after reaching 70-80% confluency, centrifuged and re-suspended in 100 µl P5 Nucleofection solution in presence of 13.5 µg/ml of corresponding siRNA. Cells were then transferred to Amaxa certified cuvettes (typically ~2 x 10⁶ cells per cuvette) and exposed to an electric shock, following program EY-100 of Nucleofector. Immediately after the shock, cells were re-suspended in pre-warmed fresh proliferation medium and plated in 6-well plates for subsequent analysis.

Oligo transfection
AONs were transfected using polyethyleneimine (PEI; ExGen 500, Fermentas, Glen Burnie, MD, USA) according to the manufacturer’s instructions. Five µL PEI/µg AON was added at a final AON concentration of 200 nM in Opti-MEM (Invitrogen, Carlsbad, CA, USA) to the myoblasts in culture. Four hours later fresh medium was supplemented to a maximum volume of 2 ml medium/well. Medium was changed 24 hours later. RNA was isolated 48 hours after start of transfection.
Chapter 2

Western blotting

Protein lysates from DM1 patient 13/800 myoblasts were prepared at the indicated time points. Wells were washed with cold phosphate buffered saline (PBS) and incubated with 100 µl RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM NaF, 1 mM Na_3VO_4, 0.1 mM Na_4P_2O_7, 0.1 mM Na_3VO_4 and Protease Inhibitory Mixture [Roche, Basel, Switzerland]) for 10 minutes on ice. Cells were disrupted by scraping to collect protein lysates and centrifuged for 10 minutes at 15,000 rpm and 4°C of temperature. Cleared lysates (supernatant) were separated on an 8% SDS/PAGE gel and transferred to PVDF (Amersham Pharmacia Biotech, Piscataway, NJ). Blots were blocked in blocking solution (5% milk in PBST 0.05% Tween20) o/n at 4°C and incubated with 1:3200 dilution in blocking solution of monoclonal 11A9 anti-AGO2 antibody (Sigma, St. Louis, MO, USA) o/n at 4°C. Then the membrane was washed 3 times in phosphate buffered saline 0.1% Tween20 (PBST) and incubated for 1 hour at room temperature in the dark with 1:10,000 dilution of the secondary antibody IRDye800 anti-Rat IgG in blocking solution. For normalization, membrane was washed again an incubated in 1:1000 dilution of β-tubulin monoclonal antibody E7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and 1:20,000 dilution of GaM680 secondary antibody. Infrared fluorescence detection and quantification of the secondary antibodies were performed using Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Average value of control samples was set at 100%.

RNA isolation

RNA from cultured cells was isolated using the Aurum™ Total RNA Mini Kit (guanidine-HCl/mercaptoethanol-based lysis, silica membrane binding; Bio-Rad), according to manufacturer’s protocol.

Northern blotting

To follow DMPK mRNA expression and breakdown fate Northern blotting was performed as described [2]. Random-primed 32P-labelled cDNAs for human DMPK (2.6 kb cDNA) and rat Gapdh (1.1 kb cDNA) were used as probes. Signals were quantified by phospho-imager analysis (GS-505 or Molecular Imager FX, Bio-Rad) and analyzed with Quantity One (Bio-Rad) or ImageJ software. Gapdh levels were used for normalization. RNA levels of control samples were set at 100%.

RESULTS AND CONCLUSIONS

Analysis by Western blot showed that AGO2 levels were reduced to less than 30% of normal levels 72 hours after AGO2 siRNA nucleofection, and this level of reduction was maintained during at least 2 more days (Figure 1A).

Cells were transfected with PS58 or PS589 (scrambled PS58 control) 72 hours after AGO2 siRNA nucleofection and RNA was isolated for DMPK analysis 48 hours later. Therefore, during the entire period of AON treatment these cells lacked more than 70% of total AGO2 protein levels (Figure 1B). RNA samples were run on a Northern blot to evaluate PS58 activity
towards DMPK transcripts (Figure 2). PS58 treatment affected DMPK expression by inducing ~25% reduction of levels from normal allele products and ~70% reduction of expanded transcripts, compared to scrambled control. This pattern of reduction was the same between cells with reduced AGO2 levels (treated with AGO2 siRNA) and cells with normal AGO2 levels (treated with control siRNA).

Figure 1. Western blot analysis of human AGO2 knock-down. (A) Blot developed by infrared detection of secondary antibodies. Green signal corresponds to antibody against AGO2, red signal to β-tubulin and protein ladder (M). The specific AGO2 bands can be seen at ~80 kD. For the quantification (see numerals below the bands), each signal was normalized with the corresponding β-tubulin band. Average values of control samples were set at 100%. (B) Quantification of AGO2 knock-down including all time points after siRNA transfection. Data from the 72 hour time point was obtained from a separate blot.
Some studies using similar siRNA SMART-pools to reduce AGO2 expression have shown that knocking-down mRNA levels of this gene by ~70% is already sufficient to impair its activity [3,4]. In fact, a study using triplet-repeat AONs designed to target the CAG repeat of HTT transcripts by the RNAi pathway also showed lack of AON activity when AGO2 expression was reduced [5]. Taking all these observations together, we conclude that RNAi is probably not involved in PS58-mediated silencing of expanded DMPK transcripts.

**Figure 2. Northern blot analysis of DMPK transcripts.** Signals corresponding to products from normal (Norm.) and expanded (Exp.) alleles are indicated by arrow heads. For the quantification (see graph), each signal was normalized to corresponding GAPDH band. Average values of control samples (Scrm: scrambled control AON) were set at 100%.
REFERENCES


CHAPTER

3

IDENTIFICATION OF CANDIDATE RIBONUCLEOPROTEINS INVOLVED IN SILENCING OF EXPANDED DMPK mRNA BY (CAG)7 2’-OME PS ANTISENSE OLIGONUCLEOTIDES

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1 Department of Cell Biology, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, The Netherlands
2 BioMarin Nederland B.V., Leiden, The Netherlands

Unpublished data
ABSTRACT

Myotonic Dystrophy type 1 (DM1) is posited to be caused by toxicity of an expanded, noncoding (CUG)n tract in mRNA transcripts from the DMPK gene. Triplet-repeat specific 2'-O-methyl phosphorothioate-modified (CAG)n antisense oligonucleotides (2'-OMe PS AONs) have been shown to efficiently reduce levels of expanded DMPK transcripts, both in vitro and in vivo. These AONs are therefore considered promising tools in therapeutic strategies for reversal of RNA toxicity in DM1. Knowledge of the mechanism of 2'-OMe PS repeat AON mediated breakdown of (CUG)n-containing RNAs is a prerequisite for the development of safe therapy for patients, for prediction of non-obvious side effects and for optimizing currently available lead compounds. Here, we present the first data from a study wherein we explored the use of the (CAG)7 2'-OMe PS AON (PS58) for the isolation of proteins that may serve as mediators of knock-down of DMPK (CUG)n RNA. Pull down of ribonucleoprotein complexes formed by association of PS58 to its target (CUG)n RNA in presence of myoblast protein extracts, followed by proteome analyses of captured complexes, helped us to identify proteins with a possible role in DMPK RNA fate specification. Among the candidate proteins identified were members of the DDX family of RNA helicases, known to control stability of expanded DMPK transcripts and proteins with intrinsic nuclease activity, including Fen1 (flap structure-specific endonuclease 1). From follow-up studies in in vitro assays with repeat RNA substrate and purified recombinant protein, we conclude, however, that FEN1 likely has no direct role in PS58-mediated breakdown of (CUG)n RNA. Other candidate proteins still await functional characterization. Availability of a list of proteins that associate with (CUG)n RNA upon PS58 binding will undoubtedly help us in further work toward mechanistic understanding of 2'-OMe PS AON-mediated breakdown of RNAs with triplet repeat expansions.
| INTRODUCTION |

Myotonic Dystrophy type 1 (DM1), is a frequent autosomal dominant disorder with highly variable and complex manifestation. Typical disease features consist of a combination of muscular, neurological and endocrine problems, each of which may vary in severity and age of onset. Problems in DM1 are caused by expansion of an unstable trinucleotide (CTG)n repeat in the 3’ untranslated region of the DMPK gene on chromosome 19q [1]. Production of long-repeat containing transcripts from this gene leads to abnormal binding of RNA splice and transport factors, like members of the MBNL family of proteins, causing factor titration and formation of anomalous ribonucleoprotein (RNP) aggregates [2-5]. In turn, these events lead to distortion in trans of alternative splicing and polyadenylation, maturation and nuclear export of numerous other mRNAs [6,7]. Also formation of homopolymeric proteins is possible, by so-called RAN translation across the repeat tract in mutant DMPK mRNAs or anti-sense transcripts produced from the same locus [8]. Altogether, these toxic events culminate into a global imbalance of proteostasis, ultimately causing cell stress with loss of physiological integrity.

Current strategies to achieve reversal of pathobiological effects in DM1 aim at complete removal of mutant DMPK mRNA or blockage of anomalous binding of proteins to the (CUG)n repeat tract. Approaches with use of repeat antisense oligonucleotides or small organic compounds that promote selective cleavage at the repeat tract and subsequent breakdown of mutant DMPK mRNA or prevent its abnormal association with members of the MBNL family of proteins have therefore been developed [9-15]. Ameliorating effects have also been reported from use of antisense oligos or shRNAs that are not repeat-selective, but promote breakdown of all DMPK mRNAs, produced from both wt and mutant DMPK alleles [16,17]. Our group has focussed on the use of 2’-O-methyl (2’-OMe) phosphorothioate (PS) modified (CAG)n AONs, to induce degradation of toxic (CUG)n transcripts [10,18]. With PS58, a 21-mer lead compound, and derivatives thereof, we achieved a 50-90% reduction in the level of DMPK (CUG)n mRNA transcripts in myoblast cells derived from the DM500 transgenic mouse model [19,20]. Additional experiments demonstrated that 2’-OMe PS (CAG)n AONs also induced a relatively fast and specific silencing of transcripts with expanded (CUG)n repeats in vitro (in DM1 patient-derived myoblasts) and in vivo (in DM500 and HSA\textsuperscript{LR} DM1 mouse models). Removal of toxic RNA also lead to normalization of aberrant pre-mRNA splicing in these models [10].

Until now, the mechanism of action underlying the silencing capacity of 2’-OMe PS (CAG)n AONs has not been fully understood (see [18] and chapter 2). This situation is in contrast to that for AONs with other types of chemistry [21], whereby breakdown of target RNAs is based on RNAse H- [11] or RNAi-mediated mechanisms [22] (although usually not experimentally proven for each individual case). Here, we followed a RNA pull-down strategy to disclose protein-based events that could be involved in binding and degradation of expanded (CUG)n DMPK transcripts by PS58. We report on the identification of several candidate proteins with a possible mechanistic role in RNA targeting and breakdown and present follow-up studies with one candidate, FEN1. The hypothetical involvement of other candidate proteins is discussed.
MATERIALS AND METHODS
Isolation of DM500 protein extract
DM500 myoblasts were derived as described [10] from a DM1 mouse model that express human DMPK (CUG)500 transcripts, the disease-causing agent in myotonic dystrophy type 1 [19]. These cells were cultured on 0.1% (w/v) gelatin-coated dishes in DMEM (GibcoBRL, Gaithersburg, MD) supplemented with 20% (v/v) FCS, 50 µg/ml gentamycin, 10 U/ml IFN-γ (BD Biosciences, San Jose, CA) and 2% (v/v) chicken embryo extract (Sera Laboratories International, Bolney, UK) at 33°C and 5% CO₂.

Protein isolation was started when DM500 myoblasts reached 90% confluency. Cells were washed twice in phosphate-buffered saline (PBS) and collected in 5 ml PBS containing 1 mM EDTA by scraping with a pliable rubber policeman. The cell pellet was collected by centrifugation at 250x g for 8 minutes and resuspended in 3 times packed cell volume (PCV) of ice-cold Hypotonic Buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, supplemented with Protease Inhibitor Cocktail at a concentration as specified by the vendor (Roche, Almere, The Netherlands)). Cells were then incubated for 15 min on ice. Aliquots were taken at different intervals to follow the loss of cell integrity by exclusion-staining with trypan blue and microscope monitoring. If cells were not completely disrupted after the incubation period, they were transferred to a glass Dounce homogenizer and homogenized on ice with 10-15 up-and-down strokes using a loose-fitting pestle. This procedure was stopped when more than 90% cells appeared trypan-blue positive and intact free nuclei became visible. To obtain the cytoplasmic fraction, the lysate was centrifuged for 15 min at 4°C and 3300x g and the supernatant was collected (nuclei are in the pellet). For the isolation of nuclear proteins, the nuclear pellet was resuspended in 200-500 µl Hypotonic Buffer to wash away possible traces of cytosolic proteins and recollected by centrifugation for 15 min at 4°C and 3300x g. The supernatant was removed and 1 PCV of ice-cold Nuclear Extraction Buffer (10 mM HEPES pH 7.9, 1 mM EDTA, 400 mM NaCl, 1 mM DTT and 1X Protease Inhibitor Cocktail (Roche, Almere, The Netherlands)) was added to the pellet. The pellet was resuspended thoroughly and incubated for 30 min on ice. The suspension was mixed by vortexing every 5 min.

Finally, cytosolic and nuclear extracts were centrifuged for 15 min at 4°C and 21,000x g to remove debris and the supernatants were collected. Glycerol was added to a final concentration of 10% (v/v). Protein concentration of the extracts was measured using a standard Bradford protocol and extracts were stored at -80°C until further use. Where indicated, cytosol and nuclear extracts were mixed to obtain a purified DM500 protein mixture of 1 mg/ml.

PS58-DMPK transcript pull-down
DMPK (CUG)90 RNA was generated by in vitro transcription of a DMPK (CTG)90 DNA template carrying flanking T7 and SP6 promoters (Supplementary Figure S1). This DMPK (CTG)90 template was generated by PCR using a human DMPK cDNA construct bearing a (CTG)90 repeat [23], T7-DMPK forward primer 5’-GAATTTAATACGACTCACTATAGGGAGAACGGGGCTCGAAGGGTA-3’ and SP6-DMPK reverse primer 5’-ATTTAGGTGACACTATAGAAGGAGAAACGGGGCTCGAAGGGGT-3’. and SP6-DMPK reverse primer 5’-ATTTAGGTGACACTATAGAAG
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GGCGTCA(G)7CAA-3', and was sequenced to verify triplet repeat number. (CUG)90 RNAs were synthesized in presence of [$\alpha$-32P]GTP, using MEGAScript® in vitro transcription kit (Applied Biosystems/Ambion, Austin, TX, USA) according to manufacturer's instructions, using 200 ng DMPK (CTG)90 template. In vitro 3'-end biotinylation was performed using the Pierce® RNA 3' End Biotinylation Kit (Thermo Scientific, Rockford, IL, USA) following manufacturer's instructions. Biotinylated DMPK (CUG)90 RNA was purified using the Aurum Total RNA Mini Kit (Bio-Rad).

For the pull down, approximately 2 μg biotin-(CUG)90 RNA was incubated with 5 μM PS58 in a total volume of 20 μl of PBS-buffered reaction mix for 5 min on ice. This mixture was then added to 100 μl pre-washed Dynabeads® MyOne™ Streptavidin T1 (Invitrogen, Oslo, Norway) and incubated on ice for 2 hours to allow binding. Beads were collected using a magnet to perform the washing steps. Samples were washed 3 times in PBS 0.1% Tween®20 (PBST) to remove excess biotin-(CUG)90/PS58 not bound to the beads. Next, 200 μl of whole cell protein extract (1 mg/mL) from DM500 myoblasts was mixed with the beads and incubated for 40 min on ice. Beads were washed 5 times with PBST. To release (CUG)90 RNA and bound proteins from the beads, beads were resuspended in 40 μl of 60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and the mixture was incubated at 95°C for 5 minutes. Beads were immediately removed by magnetic force and the supernatant containing RNA and proteins was collected, snap frozen in liquid nitrogen and stored at -80°C until further use. Proteins and RNA were separated by electrophoresis in 10% SDS/PAGE gels. Proteins were visualized by silver staining [24], whereas RNA was detected by gel exposure to X-ray film (X-Omat AR, Kodak).

Mass spectrometry

Samples were incubated at 95°C for 5 minutes and loaded on a 10% SDS/PAGE gel. Electrophoresis was carried out for 30 minutes at 80 V. The gel was stained with Coomassie Blue and each lane excised and divided with a clean scalpel in small pieces of ~1 mm³. Gel pieces were equilibrated with 50 mM ammonium bicarbonate, dehydrated with acetonitrile and dried overnight. Next day, samples were transferred to the Radboud Proteomics Center (Radboudumc) for further processing and analysis, essentially as described in [25]. Gel pieces were washed successively three times with 50 mM ammonium bicarbonate, 50% (v/v) acetonitrile and 100% (v/v) acetonitrile for 5 minutes. Gel particles were swelled in 10 mM dithiotreitol and incubated for 20 minutes at 56°C under gentle agitation to reduce protein disulfide bonds. After removal of the reduction buffer, gel particles were shrunken in 100% acetonitrile for 5 minutes at room temperature. Alkylation of reduced cysteine residues was performed by incubating the gel particles for 20 minutes in alkylation buffer (50 mM chloroacetamide in 50 mM ammonium bicarbonate) at room temperature in the dark. Following the alkylation step, gel particles were washed successively with 50 mM ammonium bicarbonate and acetonitrile three times prior to the addition of 50 μl 12.5 ng/μl trypsin in 50 mM ammonium bicarbonate (Promega) and overnight protein digestion at 37°C. Then, 50 μl 2% trifluoroacetic acid was added to each sample followed by a 20 minute incubation under gentle agitation. The supernatant was transferred to a clean tube. Remaining
proteolitical peptides in the gel pieces were recovered by shrinking the gel particles in 80% acetonitrile/0.1% formic acid for 20 minutes at room temperature under gentle agitation and subsequently the peptide-containing acetonitrile supernatant was transferred to the sample tube. The combined peptide extracts were subjected to in vacuo centrifugation to remove acetonitrile. After peptide extraction, the digested samples were loaded on STAGE-tips (Stop-And-Go Elution [26]) for clean-up, desalting and concentration prior to LC-MS analysis. The resulting peptide mixtures were analyzed by nanoflow C18 reversed phase liquid chromatography coupled online to a 7 Tesla Linear Ion Trap Fourier-Transform Ion Cyclotron Resonance mass spectrometer (LTQ FT Ultra; Thermo Fisher Scientific). The samples were measured with CID (Collision Induced Dissociation) as fragmentation technique and chromatographic separation was achieved via a linear gradient of 5% to 35% acetonitrile using 0.1% formic acid as ion pair reagent. Peptide and protein identifications were extracted from the data by means of the search program Mascot (Matrix Science). In this case, a custom RefSeq55 database was used with Mus musculus as specified taxonomy. The following modifications were allowed in the search: carbamidomethylation of cysteines (fixed), oxidation of methionine (variable) and acetylation of the N-terminus (variable). Protein identification validation was performed by an in-house developed script. Briefly, the software classifies protein identifications based on the number of uniquely identified peptide sequences and clusters proteins sharing the same set of peptides. Exponentially Modified Protein Abundance Index (emPAI) scores are used to indicate protein abundance [27]. The emPAI value is based on the correlation between the number of acquired spectra and protein abundance with a correction for the number of theoretically detectable peptides by the approach. As such, emPAI values can be compared between different proteins and samples.

**FEN1 activity assay**

To test the possible role of FEN1 activity, (CUG)90 RNA was labeled by synthesis in presence of [α-32P]GTP, using MEGAscript® *in vitro* transcription kit (Applied Biosystems/Ambion, Austin, TX, USA) according to manufacturer’s instructions. Approximately 500 ng of repeat RNA was dissolved in 50 mM Tris-HCl pH 8.0, 2 mM DTT, 30 mM NaCl, 0.1 mg/ml BSA, 5% glycerol, 2 mM MgCl\(_2\) and 4 mM ATP. PS58 was added to a final 100 μM concentration (5 μM PS58 was also tested with identical results) and incubated for 1 hour at 37°C to allow binding. Then, human purified FEN1 recombinant enzyme (ProSpec, Ness Ziona, Israel) was added to a final 100 nM concentration in 20 μl final solution and incubated at 30°C for 45 minutes. To stop the reaction, 20 μl 2X termination dye (90% formamide, 10 mM EDTA, 2X bromophenol blue) was added to the mixture.

A FEN1 substrate was used as a positive control as described [28]. 100 pmole of FEN1-cleavable oligo (downstream primer (D1) 5’-CCAAGGCCACCCGTCCACCCGACGCCACCTCTG-3’) was 5’-end labeled with 32P by T4 Kinase, by incubation for 3.5 hours at 37°C in presence of [γ32P] ATP. The reaction was stopped by heating at 72°C for 10 min. To anneal the required other oligos to the substrate, 200 μL of annealing buffer (10 mM Tris-HCl pH 8, 50 mM NaCl) was added to the mixture. 500 pmole of template primer (T1) 3’-GCTGGCACGGTGCGG
ATTTAAAGTTAGGTCGGCCAGTGGGCTGCGGTGGAGGACG-5’ was added and the reaction mix was incubated at 70°C for 5 min and then cooled down slowly to 25°C. Annealing of upstream primer (U3) was performed by adding 2 n mole of 5’-CGACCGTGCCAGCTAAATTTCAATA-3’ oligo and incubating the mixture at 37°C for 30 min. Complete FEN1 substrate was precipitated in 100% EtOH and resuspended again in annealing buffer. FEN1 substrate was assayed in parallel using the same conditions as described for (CUG)90 RNA. All samples were heated at 100°C for 5 min and run on an acrylamide/bisacrylamide (19:1 w/w, 5% for (CUG)90 RNA and 15% for FEN1 substrate) 8 M urea denaturing gels for 1 hour in MOPS running buffer at 70°C. Gels were dried and exposed to X-ray film (X-Omat AR, Kodak).

RESULTS AND DISCUSSION

Previous studies have shown that distinct proteins can be recruited specifically to an RNA target by AONs of different chemistries, triggering diverse mechanisms of RNA breakdown or modulation [29,30]. We hypothesized that PS58, by binding to expanded DMPK transcripts, will attract ribonucleoproteins (RNPs) to activate an RNA decay program in the nucleus.
or in the cytoplasm (Figure 1). Here we aimed to isolate and identify these proteins from a myoblast protein extract using a pull-down approach.

To establish a reliable procedure for protein capture and pull-down of RNP complexes several protocols for AON binding to (CUG)n expanded mRNA, incubation of AON-RNA complexes in myoblast lysate, and purification of bound proteins, were tested in pilot experiments. Use of biotinylated PS58 as bait resulted in a too high background of bound proteins in the eluate, probably due to non-specific protein binding to free 2'-OMe PS-modified AON without concurrent association to (CUG)90 RNA (data not shown). Ultimately, we chose for use of 3'-end biotinylation of the (CUG)90 RNA target, prior to hybrid formation and protein binding (Figure 2A) as the method of choice. Check-up of recovery of radiolabeled DMPK specific RNA segments that were used as bait revealed that pull down with streptavidin-coated magnetobeads was highly specific and efficient (Figure 2B lanes 3-5 versus lane 2), as anticipated. By using (CUG)n fragments of the RNA as bait and anchor, we thus certified that candidate proteins in the pull-down fraction must have been truly hybrid-bound at the moment that complexes were captured and are not proteins that only have avidity for free AON molecules.

Analysis of protein content of captured complexes by SDS-polyacrylamide gel electrophoresis and silver staining of gels enabled us to identify a distinct subpopulation of proteins with the ability to bind to biotin-(CUG)90 RNA-PS58 hybrids (Figure 2C, lane 5). Firstly, proteins binding aspecifically to beads alone (lane 1) or to beads in presence of the naked non-biotinylated (CUG)90 RNA (lane 2) could be easily differentiated and considered as background. Secondly, proteins with avidity for binding to ssRNA, as seen in the bound fractions from the mixtures that received biotin-labelled RNA, either alone (lane 3) or in presence of a control AON -with other type of chemistry and sequence- (lane 4), resulted in a very distinct profile of proteins, allowing us to identify those that were recruited to the (CUG)90 RNA-PS58 hybrid specifically (compare Figure 2C, lanes 3-5). Specific-bound proteins may be a mixture of (a) proteins that bound to free PS58 first, were shuttled by the oligo, and remained present upon hybridization-binding to the RNA and (b) proteins that bound later, i.e. those with avidity for double-stranded AON-RNA hybrids. From a mechanistic perspective, both classes of proteins in this mixture are equally interesting, so we did not adapt our pull-down isolation procedure further.

Next, proteins isolated by this pull down procedure (lanes 3, 4 and 5 in Figure 2B) were separated by polyacrylamide gel electrophoresis, excised in slices from the gel slab and subjected to proteomic analysis by mass spectroscopy. Among a total of 415 validated proteins (Figure 3), 103 (25%) were only identified in the sample wherein PS58-DMPK mRNA hybrids were formed (Supplementary Table S1). An additional 62 (15%) proteins appeared at least two-fold more abundant in the pull-down from the PS58-DMPK mRNA lysate mixture than in the mixtures that had received no AON or a control AON (Supplementary Table S2). Conspicuously, 57 (14%) proteins were pulled-down from the reaction mixture that received DMPK mRNA only, but were no longer seen when PS58 was also present. In this case, possibly these proteins associate loosely with the repeat segment in the RNA bait and are actually directly displaced by PS58 hybridization, or get detached by changes in RNA conformation, induced by PS58 binding.
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Figure 2. DMPK (CUG)90 PS58 pull-down in presence of myoblast protein extract. (A) An in vitro-synthesized DMPK RNA segment containing 90 CUG triplets was 3'-biotinylated and used as bait in presence of PS58 and myoblast protein extract. Using this approach, the aim was to identify proteins that interact specifically with the RNA AON duplex (group I of proteins) or were already bound to free PS58, prior to hybridization but remained present when duplexes were formed (II). Additionally, some proteins might bind to (CUG)n RNA independently (III). Using streptavidin-coated magnetic beads these complexes were isolated with a magnet. The washing procedure was optimized to reduce the background of proteins binding aspecifically to the beads (IV). (B) Visualization of radioactively labeled DMPK RNA that was recovered during the pull down. No RNA was used in lane 1. In lane 2, as expected, non-biotinylated RNA was not recovered. In lanes 3, 4 and 5, biotin-conjugated DMPK RNA was recovered. The intensity in lane 5 (PS58 incubation) is rather low, suggesting that PS58-mediated degradation may have occurred during the procedure. (C) Visualization of pulled-down proteins by silver staining. Proteins in the first two lanes correspond to non-specific binding to the beads (IV). Proteins in lane 3 bound to biotin-DMPK RNA only (III). In lane 4, a control AON of different chemistry and sequence was used (PS1242, 2’-F PS (CUG)7). In lane 5 (PS58 incubation), we could see some bands not present in the previous lanes, corresponding to proteins specifically recruited by PS58 bound to biotin-(CUG)90 RNA before (I) or after (II) hybridization.
As expected on the basis of similarities in electrophoresis profiles in lanes 3-5 of Figure 2B-C, a relatively large number of proteins remained present in the isolates from all mixtures, even after washing of the complexes. Altogether this group comprised 193 proteins, 46% of all proteins identified. As these proteins must represent non-specifically bead-bound proteins or proteins with a strong tendency to bind to RNA, irrespective of binding of PS58, we excluded them from further analysis. For further study we therefore focused on the remaining group of 222 proteins (54%), as we considered these the most interesting candidates for being implicated in the antisense activity of PS58.

Strikingly, although members of the MBNL family of splicing factors are centrally implicated in DM1 pathology and able to bind (CUG)n or (CCUG)n sequences [31], they were not identified in our samples. Similar studies using (CUG)n RNA as a bait for the identification of binding proteins, also failed to detect MBNL1 [32,33]. Maybe partial disassembly of the RNP complexes occurs under the salt and buffer conditions used for binding, pull-down and washing of the complexes. The inability of us and others to find MBNL proteins may thus indicate that loss of candidate proteins with selective binding properties may have occurred in the pull-down and isolation procedure, particularly for proteins that where only loosely bound in the (CUG)n RNA-AON-protein complexes.

As a first step towards the disclosure of functional clues for these candidates a gene ontology (GO) analysis was performed using String [34] and David [35,36] as bioinformatics tools. Not entirely unexpected, most of the proteins isolated from the lysate via the RNA-PS58 duplex pull-down have a role in RNA-related processes (Table 1). Furthermore, most of these proteins fitted into functional clusters in which they can strongly interact and influence each other (Supplementary Figure S2). It is thus possible that PS58 silencing activity is mediated via interference with only one or few proteins, but that this interference modulates the entire pathway in which these proteins are involved.

It was already shown in our previous studies that PS58 is able to reduce the number and size of insoluble/visible RNP foci present in the nucleus of DM1 muscle fibers [10]. Proteins with RNA helicase activity recruited by PS58 could be directly implicated in unwinding

Figure 3. Distribution of 415 proteins identified by mass spectrometry that were recovered after pull-down experiment. Four protein groups were made according to their relative abundance in control and PS58 samples.
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the hairpin structure of the long (CUG)n repeat (Figure 1B) and thus be implemented in the structural modulation of these RNP aggregates. Although this might not directly trigger a RNA degradation process, it may be a necessary first step in the silencing event (Figure 1C). It is therefore of note that we identified several members of the DDX family of RNA helicases among the group of proteins that were enriched more than two-fold by PS58 binding to the repeat (Table 2). If present in fairly high abundance we expected to see these proteins as distinct bands in the PS58 lane in Figure 2C. Indeed in this lane bands appeared at positions around 55, 70 and 83 kDa, corresponding with the predicted MWs for DDX3x, DDXy, DDX5 or DDX6. These proteins are implicated in a number of cellular processes, related to alteration of RNA secondary structure. DDX helicases have been reported to interfere with (CUG)n hairpin formation and MBNL binding [33,37], leading to foci disruption and improvement of DM1 molecular pathology, as recently reported for DDX6 [37]. Thus, to explain possible involvement in AON-mediated breakdown of expanded mRNA, we have to assume that DDX helicases have a facilitating role after AON binding by enhancing mRNA release from abnormal RNP complexes and entry in mRNA decay pathways, either in the nucleoplasm or – upon further transport – in the cytosol. Experimental follow up studies are necessary to confirm the hypothetic involvement of DDX candidates now identified.

For completion of the actual silencing by breakdown of the RNA, helicases must act in conjunction with accessory endo- or exonucleases. To substantiate this expectation and verify the validity of this idea, we specifically searched for proteins with nuclease activity. Among the candidates on our list five proteins with nuclease activity were identified: Rps3, Xrn2, Snd1, Dis3 and Fen1 (Table 3). Rps3 is a ribosomal protein for which an additional (moonlighting) role in DNA repair has been described. Involvement of this protein in the degradation of DMPK transcripts is unlikely since it does not have endonuclease

Table 1. Summary of molecular functions associated to proteins enriched in the presence of PS58.

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<tr>
<th>Cluster</th>
<th>Number</th>
<th>Percentage</th>
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<tr>
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activity for RNA [38]. Xrn2 has been found important for efficient degradation of RNA by RNase H-dependent AONs [39]. However, this protein is a 5′-3′ exoribonuclease, requiring a 5′-terminal monophosphate in the RNA for its activity [40]. Therefore, an additional protein might be required to perform the first endonucleolytic cleavage (Figure 1C) before Xrn2 can continue degrading a transcript (Figure 1D). Snd1 is a reported component of RISC [41] with a nuclease domain involved in the degradation of hyperedited (inosine modified) dsRNA [42]. However, any direct involvement in mRNA degradation has not been proven so far for this protein. Dis3 has both 3′-5′ exonuclease and endonuclease activities and it is a putative catalytic component of the RNA exosome complex [43]. This complex is responsible for proper maturation of stable RNA species such as rRNA or snRNA, for the elimination of mRNA with defects and for processing of by-products [44,45]. Although Dis3 would be an interesting candidate to pursue further, we gave its study no priority here because it was only marginally enriched in the pull-down preparation that was obtained in presence of PS58 (see Table 3).

Finally, Fen1 was recruited specifically in presence of PS58 and was completely absent in control samples (Table 3 and maybe the specific band at ~43 kDa in lane 5 of Figure 2C). Fen1, with both RNase and endonuclease activity, is involved in the removal of Okazaki fragments during DNA replication of the lagging strand [46]. During this process, when synthesis from one Okazaki fragment encounters the next, the elongating DNA strand displaces the 5′-end of the downstream segment and a non-annealed flap, containing the initiator RNA primer, is created. Fen1 is a structure-specific nuclease that participates in the removal of the flap structure, which is necessary to allow completion of lagging-strand synthesis. As an endonuclease, Fen1 specifically recognizes a double-stranded structure (RNA/DNA or DNA/DNA) with a 5′-unannealed flap (RNA or DNA) and makes a cleavage at the base [47].

We hypothesised that Fen1 might recognize, bind and cleave any branched ds AON-RNA structures that may be potentially formed upon PS58 binding to the repeat [48]. Upon Fen1-induced endonucleolytic cleavage, the mRNA might get further degraded by other exonucleases in the cell [39,49]. Such a model is supported by the observation that Xrn2,
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Table 3. Proteins enriched in the presence of PS58 which are associated with ribonuclease (RNase) or endonuclease (Endo) activity in GO analysis. Colour coding: green (high abundance) > yellow (medium abundance) > red (low abundance).

<table>
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<th>PROTEIN</th>
<th>Cluster Name</th>
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<th>PS58</th>
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<td>Snd1</td>
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a 5'-3' exoribonuclease, was recruited in PS58-DMPK mRNA hybrids at very comparable levels to Fen1. To test the potential efficacy of this putative mechanism, we incubated PS58 with the in vitro synthetized DMPK (CUG)_{90} RNA, in presence of human FEN1 enzyme (Figure 4). As a positive control for FEN1 activity, we used a combination of DNA oligos that form the required flap structure (see material and methods), which is recognized and cleaved by the enzyme [28]. Under the same conditions whereby FEN1 cleaved its natural substrate, it failed to cleave (CUG)_{90} RNA in combination with PS58. Although we do not know whether our reaction mimic the right cellular conditions needed for breakdown of the (CUG)_{90} hairpin, we consider this fairly strong evidence against an initiating role of Fen1 in PS58-mediated RNA breakdown.

Figure 4. FEN1 does not cleave (CUG)_{90} RNA substrate in presence of PS58. FEN1 is able to cleave its substrate in vitro (positive control, left panel). A similar incubation of (CUG)_{90} RNA in the presence of PS58 and FEN1 did not lead to degradation (right panel).
Concluding remarks
Since previous studies showed that the mechanism of mRNA silencing by triplet-repeat 2-OMe-PS modified AONs shares no overt features with described events in antisense technology (like splicing modulation, RNAse H mediated RNA degradation, siRNA silencing or ribozyme activity) it is clear that the working mechanism of PS58 will thus remain subject for further study.

We discard the possibility that FEN1 acts as a mediator of RNA silencing during PS58 treatment. However, the now available list of remaining candidates will help in guidance of other mechanistic studies in the future. For instance, ribosomal proteins like Rps9, Rps24, Rpl11 or Rps4x and mRNA processing proteins like Hnrnpa3, Hnrnpa1 or Hnrnpa2b1 (Supplementary Figure S2) do not have known nuclease activity, but were highly enriched in PS58-DMPK mRNA hybrids (Supplementary Tables S2 and S3). Some of these proteins could have nuclease activity not described to date or form a scaffold that attracts other proteins to build up complexes with nuclease activity. To perform tests for such speculative functions, f.e. with use of protein-RNA crosslinking assays we would ultimately need a complete new toolbox to study RNP proteins, with access to expression constructs for recombinant proteins, appropriate host cells and antibodies. These analyses go clearly beyond the scope of the work as described in this thesis chapter.

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| 18. Gonzalez-Barriga A, Mulders SAM, van de Giessen J, Hooijer JD, Bijl S, van Kessel ID, van Beers J, van Deutekom JC, Fransen JA,


**Table S1.** Proteins recruited only in presence of PS58, and absent in other conditions (continues on next page). Proteins present in PS58 sample but not present in control conditions were sorted according to abundance in the first. The cut-off score for this list was set at 0.03 (PS58 emPAI value). Colour coding: green (high abundance) > yellow (medium abundance) > red (low abundance).

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### Table S2. Proteins present in control conditions but more than 2-fold abundant in PS58 lane (continues on next page). Proteins were sorted according to fold change increase in “PS58” compared to the average of “control AON” and “no AON” values. Only proteins more than 2-fold enriched were considered. Colour coding: green (high abundance) > yellow (medium abundance) > red (low abundance).

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Candidate proteins involved in (CAG)7-mediated silencing

Table s2. (continued)

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Figure S1. Schematic representation of the DMPK construct used to generate (CUG)90 RNA for test tube experiments. Using T7 RNA polymerase RNA containing 90 CUG repeats flanked by part of the endogenous human DMPK sequence was generated.

Figure S2. String analysis for interactions between proteins enriched in the presence of PS58. The identified protein networks have defined functional clusters (surrounded by red lines). For a description of the interactions attributed between proteins (spheres), see colour key in the figure.
CHAPTER 4

WHOLE-TRANSCRIPTOME ANALYSIS OF DM1 AND CONTROL MYOBLASTS TREATED WITH A (CAG)7 ANTISENSE Oligonucleotide

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3 Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
4 Leiden Genome Technology Center, Leiden University Medical Center, Leiden, The Netherlands

Unpublished data
**ABSTRACT**

To develop a safe antisense oligonucleotide (AON) therapy for myotonic dystrophy patients, based on modulation of toxicity of the (CUG)n repeat in RNA from the mutant \textit{DMPK} gene, it is essential to get insight in the genome-wide effects that treatment with (CAG)n-AONs have on the cellular transcriptome. In the analysis of these effects we need to distinguish between transcriptome changes that result from genuine disease-correction and collateral changes evoked by \textit{in trans} side effects.

Here, we report on parallel and comparative use of RNA sequencing (RNA-Seq) and serial analysis of gene expression (SAGE) for profiling of gene expression after treatment of myoblasts with an AON consisting of seven CAG triplets, a fully 2'-O-methylribose phosphorothioate backbone and 5-methylcytosines (named “(mCAG)7” in this study). Bioinformatics analysis of the transcriptome of myoblasts from DM1 patients and unaffected individuals revealed that treatment with the (mCAG)7 AON did not affect expression of transcripts with short (CUG)n repeats (6-25 triplets). AON treatment did, however, smoothen out differences in gene expression between patients and controls. Remarkably, correction of characteristic splicing abnormalities previously reported in DM1 was not observed.
INTRODUCTION

Myotonic dystrophy type 1 (DM1) is a disease caused by a mutant expansion of a (CTG-CAG)n repeat in one of the two parental DMPK genes, associated with a toxic gain of function in the 3’-UTR of the mRNA from the mutant allele. DMPK transcripts become pathogenic (toxic), when the unstable repeat expands to above approximately 150-200 triplets. It is postulated that long (CUG)n repeats in mutant DMPK transcripts promotes abnormal binding and, ultimately, aggregation of protein factors that function in alternative RNA splicing, polyadenylation and nucleocytoplasmic transport [1], leading to global cellular ribostasis and proteostasis problems in cis and in trans. In turn, these problems cause cell stress and loss of integrity of physiological functions of many tissues and organs [2]. There is no cure for DM1, but several therapeutic strategies are currently under development [3-14]. In previous studies, we explored different aspects of use of triplet-repeat antisense oligonucleotides (AONs), which directly bind to the (CUG)n repeat of DMPK transcripts and silence their expression [14,15].

An important aspect in the pre-clinical evaluation of (CAG)n AONs is the characterization of their safety profile. AONs of this sequence can bind specifically to DMPK mRNA, but potentially also to any other transcript in the cell that carries a (CUG)n stretch. In addition, (CAG)n AONs perhaps interact with regulatory proteins that may lead to unexpected alterations in the cellular transcriptome. As a first step towards identification of – and discrimination between - undesired adverse effects and genuine effects caused by elimination of mutant DMPK transcripts, we compared whole-transcriptome profiles of DM1 and control myoblasts that were transfected with (CAG)n AONs, and performed extensive gene expression profiling (GEP) and subsequent bioinformatics analyses of the data obtained. Concomitantly, the analysis may give new clues about DM1 pathology by studying what happens with the transcription profile of these cells after reducing toxic effects of expanded DMPK transcripts.

GEP is nowadays commonly used for the characterization of activity or expression of genes in a given cell or organism, made possible by the identification and quantification of mRNA sequences. Profiles generated can be compared and analyzed to reveal differences between cell populations, indicative of relevant features like cell type, cell cycle, differentiation stage and response to stress, environment or treatments. The first widely used GEP methodology was the DNA microarray technology [16], which measures the relative expression of a predefined set of previously identified genes by hybridizing mRNA/cDNA samples to a large number of DNA sequences (probes) immobilized on a solid surface in an ordered array [17]. More recently, sequencing techniques have allowed GEP of all genes expressed in a particular specimen, not just from a predefined set. The most relevant example among the first sequencing technologies that were applied with this purpose is serial analysis of gene expression (SAGE). This method is based on the concatenation of small sequences of nucleotides, so-called tags of about 11 bp in size, derived from the 3’ part of polyadenylated mRNAs present in a given sample [18]. These tags can then be analyzed by means of high-throughput DNA sequencers. Nowadays, the advent of next generation sequencing (NGS), sometimes performed on individual RNA molecules within the entire
population, allows for whole-transcriptome characterization, giving an accurate qualitative and quantitative profile of all RNA entities present in a given sample [19]. This approach, also known as RNA sequencing (RNA-Seq), has the advantage of offering near-full sequence coverage of all transcripts present in the cell, allowing to study certain aspects that were not covered with previous technologies, such as novel alternative splice modes or antisense transcription [20].

In this study, we performed GEP by combining RNA-Seq and SAGE approaches with extensive bioinformatics analysis of the data. We monitored transcriptome changes after treating myoblasts (from unaffected individuals and patients suffering from DM1) with a (CAG)7 AON containing a fully modified 2’-O-methylribose phosphorothioate backbone and 5-methyl cytosines (named “(mCAG)7” here) or a control AON based on the scrambled sequence of (mCAG)7. This study may help to validate outcome and predict or identify any possible side effects of (CAG)n AON treatment.

<table>
<thead>
<tr>
<th>MATERIALS AND METHODS</th>
</tr>
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**Cell culture**

Myoblast cell lines from DM1 patients carrying a (CTG)760, a (CTG)800 or a (CTG)1200 repeat in the DMPK gene and control myoblast cell lines from three unaffected individuals (control 1, control 2 and control 3) were kindly provided by Dr. D. Furling (Institut de Myologie, Inserm, Paris, France) and Dr. J. Puymirat (Centre Hospitalier de l’Université Laval, Québec, Canada). Cells were cultured at 37°C and 5% CO2 in growth medium (Ham F10 medium with GlutaMAX™ (Thermo Scientific) supplemented with 20% HyClone® Bovine Growth Serum (Thermo Scientific) and 25 μg/ml gentamicin. When cells reached 100% confluence, the differentiation process was started by changing to differentiation medium (DMEM supplemented with 4 mM L-glutamine, 1 mM pyruvate, 10 μg/ml insulin [Sigma], 100 μg/ml apo-transferrin [Sigma] and 25 μg/ml gentamicin) and incubating for 7 days.

**AON treatment**

Cells were transfected in duplicate with (mCAG)7, a chemically modified AON with the sequence 5’-CAGCAGCAGCAGCAGCAGCAG-3’, containing modifications in all nucleotides: 2’-O-methylriboses, phosphorothioate backbone and 5-methyl cytosines, or with a scrambled control AON, 5’-CAGAGGACCACCAGGCCAAGG-3’, with the same chemical modifications. Transfections were performed by means of polyethyleneimine (PEI; ExGen 500, Fermentas) according to the manufacturer’s instructions. Typically, 5 μl of PEI per μg of AON was added in Opti-MEM medium (Invitrogen) to myoblasts two days before reaching 100% confluency or in differentiation medium to myotubes on day 5 of myogenesis (Fig. 1A), in both cases at a final oligo concentration of 200 nM. Fresh medium was supplemented after 4 hours and medium was changed 24 hours later. RNA was isolated at the specified time points (for Northern blotting) or 48 h after transfection in myotubes (for SAGE and RNA-Seq).
RNA isolation
RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad), following the manufacturer’s protocol with an extended on-column DNase I treatment. RNA quantity and quality was determined by RNA Nano LabChip (Agilent) measurements, for the calculation of concentrations and RNA integrity number (RIN) values per sample [21]. The concentrations were verified by spectrophotometry in a Nanodrop apparatus (Thermo Scientific). Samples were stored at -80°C until further use.

RNA sequencing (RNA-Seq)
Strand specific RNA-Seq libraries were generated using the method described by Parkhomchuk et al. [22] with minor modifications. In short, mRNA was isolated from 1 µg total RNA using oligo-dT Dynabeads (Life Technologies) and fragmented to 150-200 nt in first strand buffer for 3 minutes at 94°C. Random hexamer primed first strand was generated in presence of dATP, dGTP, dCTP and cTTP. Second strand was generated using dUTP instead of dTTP to tag the second strand. Subsequent steps to generate the sequencing libraries were performed with the KAPA HTP Library Preparation Kit for Illumina sequencing with minor modifications, i.e., after indexed adapter ligation to the dsDNA fragments, the library was treated with USER enzyme (New England Biolabs) in order to digest the second strand derived fragments. After amplification of the libraries, samples with unique sample indexes were pooled. Sequencing of the DNA fragments was performed in both directions (paired-end sequencing) using 2x100 bp read lengths on a HiSeq2000 system (Illumina), following standard manufacturer guidelines.

Serial analysis of gene expression (SAGE)
SAGE libraries were produced as described previously [23]. In short, 500 ng total RNA was hybridized to Dynabeads poly-dT magnetic beads (Invitrogen). First and second strand synthesis was performed on the beads. Bound DNA was digested with NlaIII endonuclease to leave short dsDNA segments, spanning the sequence between the most downstream NlaIII recognition site (CATG) and the poly(A) tail. Next, GEX adapter 1 was ligated and the DNA segment was digested with MmeI to create a 21 base pair fragment downstream of this adapter. This fragment was then ligated to GEX adapter 2 to complete the cassette and purified using Dynabeads streptavidin C1 magnetic beads (Invitrogen). Adaptor ligated DNA fragments were amplified by 25 cycles and PCR products were loaded on 6% Novex Tris/Borate/EDTA acrylamide gels (Invitrogen). The 96 base pair band corresponding to the complete cassette was excised and purified from the gel, followed by ethanol precipitation. Sample quality was checked on a DNA 1000 Lab-on-a-Chip (Agilent).

Sequencing was performed at the Leiden Genome Technology Center on an Illumina GA2 sequencer (Illumina). Purified samples were diluted to 10 nM and loaded on a single lane of the flow cell where, after cluster amplification, samples were put through an ultra-short 18 cycle sequencing run. Illumina Pipeline Software version 1.5 was used for data sequence processing. The FASTQ files were analysed using the open source GAPSS_B(v2) pipeline http://www.lgtc.nl/GAPSS. All sequences were trimmed to 17 base pairs to
remove the first lower quality base pair from the 3’ end of the sequences. After trimming, the NlaIII recognition site (CATG) was added to the 5’ end of the sequence to create the complete 21-22 mer nucleotide sequences. Sequences were aligned using the Bowtie short read aligner (version 0.12.7) against the UCSC hg19 reference genome, allowing for a maximum of one mismatch and a maximum of two possible positions in the genome (options: -k 1 -m 2 -n 1 --best --strata -solexa1.3-quals).

Northern blotting
To examine DMPK mRNA expression and silencing, Northern blotting was performed as described [14]. Random-primed 32P-labelled cDNAs for human DMPK (2.6 kb cDNA) and rat Gapdh (1.1 kb cDNA) were used as probes. Signals were quantified by phospho-imager analysis (GS-505 or Molecular Imager FX, Bio-Rad) and analyzed with Quantity One (Bio-Rad) or ImageJ software. Gapdh levels were used for normalization.

Data analysis and statistics
Data quality control (QC) of RNA-Seq data was assessed with the FastQC tool kit [24], giving QC information including GC content, sequence quality and overrepresented k-mers. Then, the FastX tool kit [25] was used to filter low quality data by clipping low quality bases from the reads and the FastQC tool kit was run again to assess the improvement in quality. Reads were aligned with a specialised RNA aligner, GSNAP [26]. This aligner is able to split reads that cross exon-exon boundaries. The output of this aligner was converted to a standard format (BAM) with SAMtools [27]. To exploit the strand-specific nature of the data, the read pairs originating from the transcripts located on the forward strand were separated from those located on the reverse strand. After data splitting, BEDtools was used to count numbers of reads mapping to individual exons [28]. Gene expression and transcript assembly analysis was done by Cufflinks [29]. Then, for later downstream analysis, a list of exact absolute counts per exon was generated.

Analysis of SAGE data was done using the R programming package [30]. Data was filtered for at least 0.5 counts per million, in at least half of the samples. To perform the comparison between groups we used the libraries “limma”, “edge R”, “org.Hs.eg.db” and “biomaRt”. R was also used to generate the volcano plots and the multidimensional scaling (MDS) analysis of the RNA-Seq data. Specific threshold limits (0.05 for the q-value and 2 for the fold change) were applied to generate the volcano plots, using the library “metabolomics” in addition to the ones previously mentioned. The MDS analysis was performed using the “edge R” library. MDS represents the samples on a two-dimensional scatterplot according to the pairwise expression distances of the typical log2 fold changes of the top 100 genes that best distinguish the samples.

To gain further insight into the biology behind certain genes subsets in our dataset, the Gene Set Enrichment Analysis (GSEA) method was used to compute overlap with gene ontology signatures and test whether they were significantly enriched [31].

For all these analyses, either p-value or q-value functions were used (indicated in each experiment) for the estimation of statistical significance. The q-value is the false discovery rate analog of hypergeometric p-value after correction for multiple hypothesis testing [32].
RESULTS

Evaluation of DMPK silencing after (mCAG)7 AON treatment

We used six different myoblast cell lines (three from DM1 patients and three from unaffected controls) to perform gene expression profiling by RNA-Seq and/or SAGE (Table 1). Cells were analyzed after seven days of differentiation to myotubes in order to achieve a transcriptome profile that optimally matched the profile of adult muscle. Since we were interested in possible changes of gene expression caused by (mCAG)7 AON treatment that may arise already at myoblast stage but also have consequences for later steps in myogenesis, we used a protocol with first transfection before onset of differentiation (Fig. 1A). To monitor DMPK silencing during the entire course of differentiation, we performed Northern blot analysis of RNA samples obtained at day -1 (one day before onset of differentiation) and at days 1, 3 and 5 after medium switch for differentiation (Fig. 1A). Hybridization with a 32P-labeled DMPK probe revealed that strong silencing of expanded DMPK transcripts occurred in myoblasts before the medium switch for differentiation and that silencing was stably maintained in myotubes during the initial five days of differentiation (Fig. 1B). At day 5, cells did undergo a second round of transfection to make sure DMPK levels would remain low until day 7, the time point used for RNA-Seq and SAGE analysis. As expected, we could confirm a significant reduction in DMPK transcripts in the DM1 cell line, whereas DMPK transcripts of the control cell line appeared not affected (Fig. 1C, note that these two methods cannot discriminate between expanded and normal-sized allele products). We performed additional SAGE analysis in the other two DM1 and control cell lines (Table 1). In all cases comparable DMPK silencing effects were observed in DM1 cells, with no effects on DMPK expression seen in controls (Fig. 1D).

Effects of (mCAG)7 treatment on expression of (CTG)n-repeat containing genes

One anticipated but undesired effect of (mCAG)7 treatment would be silencing of other (CTG.CAG)n-repeat containing genes than DMPK that produce transcripts with a (CUG)n

Table 1. Cell lines used in this study. Overview of control and DM1 myoblast cell lines including DMPK (CTG)n repeat lengths. The transcriptome profiling technique(s) used for analysis of each cell line are given in the last column.

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repeat sequence, of which there are several in the human genome. For a complete listing of these genes see refs. [14,33]. Here, we studied expression of fourteen representatives of this group of genes with repeats of 6 to 25 (CTG.CAG) triplets (Table 2). Very encouragingly, we found that only DMPK was consistently silenced in all DM1 cell lines. Other (CUG)n-containing transcripts were not or only mildly affected, whereby no consistent effects across all cell lines were seen (e.g. LTBP3 and STEAP3). Importantly, there was no correlation between the level of reduction and (CUG)n repeat length for the repeat range (6-25 CUG triplets) analyzed (Fig. 2).

Transcriptome effects of (mCAG)7 AON treatment
To obtain better insight in the overall effects of (mCAG)7 AON activity we first made a comparison between the transcriptomes of DM1 and control myotubes that received treatment with (mCAG)7 or with the scrambled control AON (Fig. 3). RNA-Seq data analysis revealed 266 genes whose expression changed significantly after (mCAG)7 treatment in the (CTG)800 cell line (q<0.05 threshold; 113 upregulated and 153 downregulated) and 484 that had undergone expression change in the control cell line 1 (q<0.05 threshold; 412 upregulated and 72 downregulated). SAGE analysis also showed statistically significant changes in gene expression of the (CTG)800 cell line after (mCAG)7 treatment (q<0.05 threshold; 452 genes: 293 upregulated and 159 downregulated), but not in control cell lines. Comparing these three lists of genes, there was an overlap of four genes that were significantly dysregulated in all datasets after (mCAG)7 treatment (INHBE, MYL4 and EGR2 with enhanced expression; TMEM158 with reduced expression). SAGE expression analysis in the other cell lines (the three control cell lines, (CTG)760 and (CTG)1200) showed no statistically significant change for any gene. Given that RNA-Seq and SAGE analysis were powered in the same way for all cell lines (n=2), we conclude that RNA-Seq is more sensitive and thus provides a better ability to detect significant changes in gene expression.

Next, we attempted to deconvolute complexity in our dataset, needed because changes in gene expression observed in some cell lines could be caused by either (i) unspecific cellular
Transcriptome analysis of cells treated with (CAG)7

A

Proliferation >>

100% confluent myoblasts

Differentiation >>

Start differentiation (day 0)

day -4

AON Transfection

day -3

day -2

day -1

day 1

day 2

day 3

day 4

day 5

day 6

day 7

AON Transfection

B

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<td>% Normal</td>
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C

![Graph showing DMPK expression levels](image)

D

![Bar graph showing DM1 and control lines](image)
Table 2. Expression analysis of genes containing a (CTG)n repeat (n>5). Several transcripts with more than 5 CUG triplets were included in the analysis. *(CTG)n: number of CTG triplets in the gene. Please note that the number of CTG triplets indicated (given as variable for DMPK) for each gene does not indicate a fixed repeat length. Most repeats occur as polymorphic DNA segments and occur with variable length throughout the human population. Loc.: location of the repeat in the gene. If the repeat is part of the coding sequence (CDS), the exon number containing the repeat is given, followed by the total amount of exons in the gene between brackets. F.C.: fold change of expression between (mCAG)7 AON and scrambled control. Sig.: statistical analysis of the comparison indicating p value/q value. The q value is adjusted for the total amount of comparisons. Comparisons were considered significant when (p or q)<0.05: *(p or q)<0.05, **(p or q)<0.01, *** (p or q)<0.001 and, in those cases, corresponding values are highlighted in bold. Assay: color key used to indicate the type of assay used for RNA analysis; yellow indicates RNA-Seq, blue indicates SAGE.

<table>
<thead>
<tr>
<th>Gene</th>
<th>(CTG)n</th>
<th>Loc.</th>
<th>F.C. 3</th>
<th>Sig. 3</th>
<th>F.C. 4</th>
<th>Sig. 4</th>
<th>Assay 3</th>
<th>Sig. 3</th>
<th>F.C. 3</th>
<th>Sig. 4</th>
<th>Assay 3</th>
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</thead>
<tbody>
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<td>DMPK</td>
<td>Variable 3'UTR</td>
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<td>*/n.s.</td>
<td>0.18</td>
<td>*/n.s.</td>
<td>0.67</td>
<td>*/n.s.</td>
<td>RNA-Seq</td>
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<td>n.s/n.s.</td>
<td>0.80</td>
</tr>
<tr>
<td>TCF4</td>
<td>25 Intron 3</td>
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<td>n.s/n.s.</td>
<td>1.14</td>
<td>n.s/n.s.</td>
<td>0.55</td>
<td>*/n.s.</td>
<td>SAGE</td>
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<td>n.s/n.s.</td>
<td>1.06</td>
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<tr>
<td>RPL14</td>
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<td>0.43</td>
<td>*/n.s.</td>
<td>0.67</td>
<td>*/n.s.</td>
<td>SAGE</td>
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<td>n.s/n.s.</td>
<td>0.94</td>
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<tr>
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<td>15 Intron 1</td>
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<td>n.s/n.s.</td>
<td>1.61</td>
<td>n.s/n.s.</td>
<td>1.11</td>
<td>n.s/n.s.</td>
<td>RNA-Seq</td>
<td>1.03</td>
<td>n.s/n.s.</td>
<td>1.11</td>
</tr>
<tr>
<td>NOTCH4</td>
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<td>1.13</td>
<td>n.s/n.s.</td>
<td>1.21</td>
<td>n.s/n.s.</td>
<td>1.27</td>
<td>n.s/n.s.</td>
<td>SAGE</td>
<td>1.27</td>
<td>n.s/n.s.</td>
<td>0.88</td>
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<tr>
<td>LRP8</td>
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<td>n.s/n.s.</td>
<td>0.77</td>
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<td>RNA-Seq</td>
<td>0.92</td>
<td>n.s/n.s.</td>
<td>1.72</td>
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<tr>
<td>MAP3K4</td>
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<td>n.s/n.s.</td>
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<td>RNA-Seq</td>
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<td>n.s/n.s.</td>
<td>0.75</td>
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<td>n.s/n.s.</td>
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<td>n.s/n.s.</td>
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<td>n.s/n.s.</td>
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<td>n.s/n.s.</td>
<td>0.91</td>
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<td>0.91</td>
<td>n.s/n.s.</td>
<td>1.09</td>
<td>n.s/n.s.</td>
<td>RNA-Seq</td>
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<td>n.s/n.s.</td>
<td>0.71</td>
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<td>EIF2AK3</td>
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<td>n.s/n.s.</td>
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<td>n.s/n.s.</td>
<td>RNA-Seq</td>
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<td>n.s/n.s.</td>
<td>0.71</td>
</tr>
<tr>
<td>CHRNA3</td>
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<td>1.06</td>
<td>n.s/n.s.</td>
<td>0.98</td>
<td>n.s/n.s.</td>
<td>1.34</td>
<td>n.s/n.s.</td>
<td>RNA-Seq</td>
<td>1.20</td>
<td>n.s/n.s.</td>
<td>1.25</td>
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<tr>
<td>RBM23</td>
<td>6 12(14)</td>
<td>1.16</td>
<td>n.s/n.s.</td>
<td>0.63</td>
<td>n.s/n.s.</td>
<td>1.06</td>
<td>n.s/n.s.</td>
<td>RNA-Seq</td>
<td>0.99</td>
<td>n.s/n.s.</td>
<td>1.66</td>
</tr>
<tr>
<td>STEAP3</td>
<td>6 Intron 1</td>
<td>0.89</td>
<td>n.s/n.s.</td>
<td>0.63</td>
<td>n.s/n.s.</td>
<td>0.91</td>
<td>n.s/n.s.</td>
<td>RNA-Seq</td>
<td>0.94</td>
<td>n.s/n.s.</td>
<td>0.99</td>
</tr>
</tbody>
</table>

response to triplet-repeat AONs and/or (ii) downstream effects of reduction of expanded DMPK gene expression (only applicable to DM1 cell lines) or superposition of these effects.

As we may assume that non-specific side effects (i) occur in both the control and DM1 cell populations, we took RNA-Seq data of these cells to compare the genes that significantly changed expression with (mCAG)7 treatment in both (CTG)800 and control cell line 1. Out of the total set of genes that had undergone expression change (see Fig. 3), altogether 27 genes were identified that showed congruent changes in expression in both cell lines (Table 3). Twenty genes appeared upregulated (p value of overlap < 1.015e-14) and 7 were downregulated (p value of overlap < 4.763e-07). From this list of genes, only six were
Transcriptome analysis of cells treated with \((\text{CAG})_7\)

Figure 2. \((\text{mCAG})_7\) AON activity in relation to target repeat length between 6 and 25 CUG triplets. The graphs show a linear regression analysis of \((\text{mCAG})_7\) AON effect versus number of CUG triplets in the target RNA as analyzed by (A) RNA-Seq for one control and one DM1 cell line or by (B) SAGE for three control and three DM1 cells (merged). No significant correlations were found \((\alpha=0.05)\) using either Spearman or Pearson tests.

We next attempted to identify genes that underwent specific change in expression – directly, or indirectly - in response to knock-down of toxic \(\text{DMPK}\) transcripts. We therefore specifically searched for genes that did undergo expression change in the \((\text{CTG})_{800}\) cell line upon treatment with the \((\text{mCAG})_7\) AON, in the entire RNA-Seq and SAGE dataset (Fig. 3). Out of a total of 75 genes which showed change in both the RNA-Seq and SAGE analysis, 45 were significantly upregulated \((p\text{ value of overlap } < 3.389\times10^{-56})\) and 30 were significantly downregulated \((p\text{ value of overlap } < 1.868\times10^{-35})\) in both data sets. We
Chapter 4

Figure 3. Genes whose expression was significantly changed by (mCAG)7 AON treatment: comparison between different cell groups and techniques. Analysis was performed separately for upregulated and downregulated genes. The threshold to include a gene in this analysis was $q<0.05$ (for the comparison (mCAG)7 AON treatment versus scrambled AON treatment). In DM1 cell lines, the overlap of these genes between RNA-Seq and SAGE genes was statistically significant, both in upregulated and downregulated genes (note that there were no significant gene expression changes detected by SAGE for the control cell line, so no overlap could be tested between techniques in that case. When comparing genes with overlap between DM1 and control cell lines, there was also significant overlap in both upregulated and downregulated genes. The genes that were significantly upregulated or downregulated in both control and DM1 cell line (as determined by RNA-Seq) are depicted in Table 3. Asterisks indicate whether the overlap between gene sets in the intersection between pairs of circles is significant (i.e. not representing the result of random intersection **: $p<0.01$, ***: $p<0.001$). Note that three upregulated genes and one downregulated gene appear at the intersection of the three circles for upregulated and downregulated genes respectively, and thus they are also included in each comparisons between gene sets.

performed a Gene ontology (GO) analysis of these genes using a Gene Set Enrichment Analysis (GSEA) tool [31]. When looking at genes that were upregulated after (mCAG)7 treatment in the DM1 cell line, GSEA found a significant enrichment ($q=1.05E-09$) of eight genes involved in development of skeletal muscle or myogenesis (ACTC1, PPFIA4, COL15A1, MYL4, DES, MYOG, KLF5 and TNNT1). GSEA analysis of genes downregulated by AON treatment resulted in a significant enrichment ($q=1.71E-07$) of six genes involved in epithelial-mesenchymal transition (TFPI2, MMP1, FBLN1, FMOD, ANPEP and SFRP1).
Table 3. RNA-Seq analysis of genes whose expression changed significantly in both control and DM1 cell lines by (mCAG)7 AON treatment. List of genes that are significantly upregulated or downregulated (q<0.05) in both DM1 and control cell line. *: genes indicated in bold lettering showed significant change in expression in both cell lines (as determined by RNA-Seq) and in addition were also significantly changed in the SAGE data (see triple overlap of the circles in Fig. 3).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>DM1 cell line</th>
<th>Control cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAS1R3</td>
<td>Taste receptor, type 1, member 3</td>
<td>6.687</td>
<td>4.469</td>
</tr>
<tr>
<td>UCKL1-AS1</td>
<td>Uridine-cytidine kinase 1-like 1 Antisense</td>
<td>3.179</td>
<td>2.391</td>
</tr>
<tr>
<td>FBXO39</td>
<td>F-box protein 39</td>
<td>2.565</td>
<td>2.464</td>
</tr>
<tr>
<td>ZNF619</td>
<td>Zinc finger protein 619</td>
<td>2.473</td>
<td>2.177</td>
</tr>
<tr>
<td>C19orf35</td>
<td>Chromosome 19 open reading frame 35</td>
<td>2.468</td>
<td>1.975</td>
</tr>
<tr>
<td>MMRN2</td>
<td>Multimerin 2</td>
<td>2.216</td>
<td>2.221</td>
</tr>
<tr>
<td>TREX1</td>
<td>Three prime repair exonuclease 1</td>
<td>2.055</td>
<td>2.810</td>
</tr>
<tr>
<td>INHBE *</td>
<td>Inhibin, beta E</td>
<td>1.648</td>
<td>1.451</td>
</tr>
<tr>
<td>SLC8A2</td>
<td>Solute carrier family B (sodium/calcium exchanger)</td>
<td>1.611</td>
<td>2.426</td>
</tr>
<tr>
<td>UCP3</td>
<td>Uncoupling protein 3 (mitochondrial, proton carrier)</td>
<td>1.572</td>
<td>1.560</td>
</tr>
<tr>
<td>MYOM3</td>
<td>Myomesin 3</td>
<td>1.549</td>
<td>1.298</td>
</tr>
<tr>
<td>EGR2 *</td>
<td>Early growth response 2</td>
<td>1.518</td>
<td>1.266</td>
</tr>
<tr>
<td>RPPH1</td>
<td>Ribonuclease P RNA component H1</td>
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<td>CSNK1D</td>
<td>Casein kinase 1, delta</td>
<td>1.437</td>
<td>2.157</td>
</tr>
<tr>
<td>KRT7</td>
<td>Keratin 7</td>
<td>1.422</td>
<td>1.312</td>
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<tr>
<td>MYBPH</td>
<td>Myosin binding protein H</td>
<td>1.405</td>
<td>1.231</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
<td>1.306</td>
<td>1.278</td>
</tr>
<tr>
<td>MYL4 *</td>
<td>Myosin, light chain 4, alkali; atrial, embryonic</td>
<td>1.296</td>
<td>1.336</td>
</tr>
<tr>
<td>TRIB3</td>
<td>Tribbles homolog 3 (Drosophila)</td>
<td>1.260</td>
<td>1.383</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Vascular cell adhesion molecule 1</td>
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<td>1.769</td>
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<tr>
<td>ALDH1A3</td>
<td>Aldehyde dehydrogenase 1 family, member A3</td>
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<td>0.726</td>
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<tr>
<td>TEMEM158 *</td>
<td>Transmembrane protein 158</td>
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<td>0.742</td>
</tr>
<tr>
<td>CIT</td>
<td>Citron (rho-interacting, serine/threonine kinase 21)</td>
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<td>0.643</td>
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<td>Integrin, alpha 10</td>
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<td>0.613</td>
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<td>AMH</td>
<td>Anti-Mullerian hormone</td>
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<td>0.7842</td>
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<td>Neuronal pentraxin</td>
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<tr>
<td>CD79A</td>
<td>Molecule, immunoglobulin-associated alpha</td>
<td>0.389</td>
<td>0.324</td>
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</table>

Reversal of DM1 expression pattern by (mCAG)7 treatment

The finding that a significant portion of genes that were upregulated after (mCAG)7 treatment are involved in myogenesis triggered us to hypothesize that expanded DMPK silencing could have reverted (at least in part) the downstream effects of the repeat in gene expression, and consequently improving myoblast differentiation of the DM1 cell line. To provide a more
solid theoretical foundation for this hypothesis, we first tried to characterize the differences in expression between DM1 and control cell lines (independently of (mCAG)7 treatment) to generate a characteristic DM1 expression pattern of the cells used in our experiment. To do so, we analyzed differences in gene expression of the (CTG)800 cell line compared to a control cell line in the RNA-Seq data (Fig. 4A). For a q<0.05 threshold, we found 3339 genes that differed more than two fold in expression between both cell lines. Since we are not using isogenic cell lines, we have to consider that some of these differences in gene expression may be due to genetic background differences between both cell lines, and not only are due to the pathogenic condition. Making a similar comparison with the SAGE data for the DM1 and control cell lines, we found 618 genes with significant different expression (this lower number of genes found by SAGE can be taken as an additional indication of the superior sensitivity of RNA-Seq). A significant portion of these genes (42%) overlapped with the list of genes found in the RNA-Seq analysis (p value of overlap < 7.152e-64).

We then continued with the analysis of the DM1 expression pattern found in the RNA-Seq data. From the 3339 genes with significant expression differences between (CTG)800 and control cell line, 2294 genes showed at least a >2-fold higher expression and 1045 genes showed at least <50% lower expression in DM1 compared control cells, respectively. GSEA analysis of overexpressed genes indicated a significant enrichment for pathways related to inflammatory response (50 genes, q=7.08E-23), with overlap for groups of genes that respond to signaling of certain cytokines, like IFNγ (88 genes, q=1.65E-62), IFNa (55 genes, q=3.36E-47), NFκB/TNFα (69 genes, q=4.28E-41) and STAT5/IL2 (51 genes, q=1.1E-23), and genes for complement system proteins (58 genes, q=4.88E-30). Other relevant clusters in our list of overexpressed genes were for genes involved in apoptosis (38 genes, q=5.99E-17) and epithelial-mesenchymal transition (75 genes, q=2.21E-47). Groups of genes whose activity is lower in DM1 cells were significantly enriched for functioning in myogenesis (73 genes, q=1.23E-67) and response to KRAS activation (26 genes, q=5.30E-12).

Upon (mCAG)7 treatment, the number of overexpressed genes was reduced (from 2294 to 1580) and the total number of underexpressed genes hardly changed (1045 vs 1138) (Fig. 4A). To us, the pattern of DM1 gene expression in the volcano plots, for both overexpressed and underexpressed genes, appeared more similar to control cells upon (mCAG)7 treatment, i.e. closer to Log2 fold expression difference = 0 (compare left and right graph of Fig. 4A). To verify this observation, we performed a classification of the genes according to the direction of expression change upon (mCAG)7 treatment (Fig. 4B). We inferred that out of a total of 2294 genes that showed differential overexpression originally, 1039 genes (45%) showed a lower fold change in expression difference (i.e. had undergone partial normalization towards control-like expression); 93 (4%) underwent further upregulation and 1162 (51%) remained unchanged. Out of the group of underexpressed genes in DM1 cells (1045 genes altogether), 315 genes (30%) underwent upregulation as an effect of AON treatment, 95 (9%) were further downregulated and 635 (61%) did not change their expression. Therefore, in both cases, the majority of genes that did change, adapted their expression into the direction of the normal expression pattern, as found for the control cell line. It should be noted that, for some of these genes, although the fold change in
Transcriptome analysis of cells treated with (CAG)7

Figure 4. Partial correction of DM1 expression pattern after (mCAG)7 treatment as determined by RNA-Seq. (A) Volcano plots of gene expression. Genes in the (CTG)800 cell line are represented by dots plotted in comparison to their expression in the control cell line by fold change (x-axis) and significance (y-axis). Genes with q<0.05 and >2-fold change are indicated in blue (downregulated in DM1) or red (upregulated). Left graph shows the expression pattern after transfection with the scrambled control AON (DM1 signature), whereas right graph shows the expression pattern after transfection with (mCAG)7 AON ((mCAG)7 treatment). (B) Pie-charts representing the distribution of genes according to their change in expression after (mCAG)7 treatment. Genes that changed their expression >25% away from their value in the control cell line account for the dark grey area of the chart, genes that maintain the same levels (±25%) are included in the light grey sector and genes that change their expression >25% towards their value in the control cell line are represented in the green sector. (C) Representation of average fold-change between control and DM1 cells for the entire population of genes. The fold change is significantly lower in cells transfected with (mCAG)7 AON (treatment) compared to those transfected with the scrambled control AON (signature). ***: p<0.001. (D) Multidimensional scaling analysis of FPKM values of the entire dataset of genes detected in the RNA-Seq experiment. (E) Representation of expression differences for a selection of genes in DM1 cells whose levels were significantly closer to the healthy control after transfection with (mCAG)7 AON. *Genes that were expressed in the DM1 cell line but not in the control cell line were given an arbitrary log2FC value of 6.64 (FC=100). **Genes that were expressed in the control cell line but not in the DM1 cell line were given an arbitrary log2FC value of -6.64 (FC=0.01). These genes are described in Table 4.
expression was reduced, the deviation in their expression remained statistically significant. Nevertheless, the average magnitude of change came significantly closer to control cells (reference value of zero) after (mCAG)7 treatment, as assessed by comparing the mean fold change of expression difference between cell lines under scrambled AON treatment versus (mCAG)7 treatment (Fig. 4C). This was further confirmed by a multidimensional scaling analysis, which uses the most representative genes of each sample group to create a representative signature of the expression pattern (Fig. 4D).

To verify our expectation that the genes that recovered expression towards levels as found in the control cell line belong to the same group as those that were found dysregulated in DM1, we performed a new GSEA analysis with the genes whose expression level was partially or completely corrected by (mCAG)7 treatment (examples from these genes are depicted in Table 4 and Fig. 4E). Indeed, we found that a significant portion of the genes with reduced expression after (mCAG)7 treatment were also those that were found overexpressed in the previous analysis, i.e. the genes with a role in inflammatory responses (q=1.32E-21), IFNγ response (80, q=8.24E-80), IFNα response (55 genes, q=8.00E-66), NFκB/TNFα signaling (45 genes, q=2.36E-32), STAT5/IL2 signaling (30 genes, q=1.05E-16), complement biology (43 genes, q=3.70E-30), apoptosis (27 genes, q=2.08E-16) and epithelial-mesenchymal transition (40 genes, q=7.08E-27). Similarly, genes that were underexpressed in DM1, but recovered expression towards control levels with (mCAG)7 treatment, were again found significantly enriched for functioning in myogenesis (11 genes, q=2.10E-06) and expression induced by KRAS activation (12 genes, q=4.09E-07). This indeed confirms that genes that were found dysregulated in DM1 are also in majority the ones that recovered their expression to control values upon (mCUG)7 treatment.

One of the best characterized hallmarks of DM1 is the incorrect splicing that several gene transcripts undergo as a consequence of MBNL and CUGBP1 dysregulation [34,35]. We looked into the RNA-Seq data at a panel of genes reported to be incorrectly spliced in DM1 [36]: CLCN1 (exon 7), DMD (exon 78), TTN (exon 5), LDB3 (exon 7), NFIX (exon 7), PHKA1 (exon 18), INRS (exon 11) MBNL1 (exon 7), BIN1 (exon 11), SERCA1 (exon 22) and NCOR2 (exon 45). From all these genes, exon coverage was only sufficient to perform splicing analysis for BIN1, NCOR2, MBNL1 and NFIX (Fig. 5). The splicing pattern on BIN1, NCOR2 and MBNL1 was indeed significantly different between control and DM1 cells but, strikingly, there was no correction by (mCAG)7 AON treatment. Inclusion of exon 7 of NFIX was not affected in our DM1 cells as compared to the control.

| DISCUSSION |

This study aimed to monitor transcriptome changes that occurred in healthy and DM1 myoblasts upon transfection with a (mCAG)7 AON. A gene expression profiling (GEP) analysis based on RNA-Seq and SAGE-based quantitation and bioinformatics analysis of data was conducted to reveal different types of changes: (i) effects on the target transcripts for which the (mCAG)7 AON was intended, i.e. (CUG)n-expanded DMPK mRNA isoforms, (ii) effects on (CUG)n-containing mRNAs from other genes in the genome, (iii) collateral effects of AON treatment on gene expression, not necessarily due to direct hybridization activity of
the AON, and (iv) expression changes that may be indicative for normalization of the genetic disease expression pattern upon silencing of the mutation (pathology reversal). The findings obtained can potentially serve to provide better insight in the type and magnitude of side effects of the oligo and also to understand the extent of reversal to a normal myoblast-myotube transcriptome expression pattern that can be achieved upon treatment of DM1 cells with this AON.

**Table 4.** Genes with corrected expression after (mCAG)7 AON treatment. List of genes with their log2 fold change (FC) expression in DM1 cells respective to control cells after transfection with scrambled AON (signature) or (mCAG)7 AON (treatment). *Genes that were expressed in the DM1 cell line but not in the control cell line were given an arbitrary log2FC value of 6.64 (FC=100). *Genes that were expressed in the control cell line but not in the DM1 cell line were given an arbitrary log2FC value of -6.64 (FC=0.01). The expression changes of these genes are represented in Fig. 4E.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Log2 fold change</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Syndecan Binding Protein (Syntenin) 2</td>
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The first obvious transcript to study was DMPK itself. We observed a significant reduction in DMPK RNA in all DM1 cell lines by both RNA-Seq and SAGE, but DMPK expression was not affected by treatment with (mCAG)7 AON in unaffected myoblasts. Moreover, we could show by Northern blotting that DMPK reduction in DM1 myoblasts corresponded specifically to the mutant products of the expanded allele.

In addition to DMPK, several other transcripts containing a small (CUG)n repeat occur in the transcriptome. None of these potential targets for (mCAG)7 appeared significantly silenced by the oligo. Furthermore, there was no significant correlation between the number of CUG triplets in the target and the level of reduction for a range between 6 to 25 triplets. Taking these findings together, we conclude that the impact of (mCAG)7 treatment on transcripts containing repeats in this range is negligible. This fits with our earlier observation.
that (CAG)7 AONs works with selectively towards products of the mutant DMPK allele (containing >25 CUG triplets) leaving normal transcripts with ~5-15 CUG triplets largely unaffected (see Chapter 2 [15] and Fig. 1B).

Other potential side effects of (mCAG)7 treatment may not be dependent of direct binding to transcripts with (CUG)n repeats. For instance, (mCAG)7 could interact with cellular factors and trigger unexpected downstream gene expression responses. To explore this possibility, we looked at significant changes in global gene expression in control cell lines, again by comparing treatment with (mCAG)7 and scrambled AON. We took control cell lines instead of DM1 cell lines for this analysis to avoid identification of any changes that were directly or indirectly repeat-related. When looking at the fold change in gene expression between (mCAG)7 and scrambled AON treatment, we could only find significantly dysregulated genes in control cell line 1 by RNA-Seq, but not by SAGE in this one nor any of the two other control cell lines. We can exclude that the expression change of these genes is a downstream effect of DMPK silencing since we demonstrated that DMPK expression levels were not reduced in control cell line 1 upon AON treatment.

Gene expression differences between (mCAG)7 and scrambled AON treatment in (CTG)800 DM1 cells revealed that twenty-seven genes changed expression as in the control cell line. However, only eight (out of a total of 1536 genes analyzed in both cell lines) showed more than two-fold change in expression, six of them being upregulated (Taste Receptor (TAS1R3), Uridine-Cytidine Kinase (UCKL1), F-Box Protein 39 (FBXO39), Zinc Finger Protein 619 (ZNF619), Multimerin 2 (MMRN2) and Three Prime Repair Exonuclease (TREX1)) and two of them downregulated (Neuronal Pentraxin (NPTX1) and CD79a Molecule (CD79A)). These transcripts belong to very different functional classes of genes, according to our GSEA analysis, so it is difficult to give any statements on causal associations between (mCAG)7 treatment and effects on their expression. Moreover, some of these genes, like TAS1R3, NPTX1 or CD79a, are not characteristic for myoblasts or muscle tissue and were indeed found to be expressed at a very low level. These observations led us to think that, at least for some of these genes, the changes observed (even when corrected for the false discovery rate) may be due in large part to stochastic variation in gene expression (culture or experimental context-dependent; perhaps under epigenetic regulation) rather than to a true biological effect. Taking together, the SAGE and RNA-Seq analyses yielded no evidence to support that (mCAG)7 AON induces unspecific off-target changes in the transcriptome or that these changes could lead to detrimental effects in the cell. Further study is needed to substantiate this conclusion.

The key issue in the study described here is finding an answer on the question whether (mCAG)7 treatment could help restoring the DM1 transcription pattern to levels observed in control cell lines. To do just that, we generated a characteristic DM1 expression pattern profile of the transcriptome by comparing gene expression in DM1 cells with that in control cells (fold change and significance) in samples that were transfected with the scrambled control oligo. By performing GO analysis of genes whose expression was significantly different, we identified classes of genes involved in stress responses and inflammation, like apoptosis, complement activation, interferon response and cytokine signalling, including
TNFα, IL2 and IL6. Other transcriptome studies in DM1 have also reported overexpression of genes related to immune response and interferon activation [37]. Specifically, cytokines like TNFα and IL6, are known to be elevated in DM1 patients [38,39], probably due to the toxic effects exerted by the mutant (CUG)n expansion [40,41]. In addition, genes related to epithelial-mesenchymal transition were also overexpressed, whereas genes related to myogenesis were underexpressed. This could indicate a partially disrupted differentiation program in DM1 cells compared to healthy myoblasts [42], which may also be due to detrimental effects of the DMPK mutation as has been reported by others [43,44]. These observations indicate that indeed many of the genes in the DM1 cell line may show dysregulated expression due to the pathogenic condition of the cells.

Equally interesting and important from a therapeutic perspective, is the finding that (mCAG)7 AON treatment partially corrected abnormal gene expression in DM1 patient myotubes. Among the responsive genes, we found several related to interferon and inflammation biology. For instance, IFI27L2, BATF2, IFI35, MX2, IRF1 and CD40 were overexpressed in DM1 cells, but expressed at the level of control cell line levels after (mCAG)7 treatment (see Table 4). Also the cluster of genes upregulated by IL6 showed expression correction. Likewise, genes related to myogenesis that we found underexpressed in DM1, like PTGIS, MYOZ1, MSTN and MYL10, partially recovered normal expression levels (see Table 4). Although our results are encouraging and provide new leads for study of DM pathology, we should be cautious with drawing too definite conclusions from this exploratory study, since most of the findings discussed here need to be validated by alternative techniques like qPCR and be corroborated in additional cell lines and experiments.

Commonly, aberrant splicing is considered by most researchers in the field as central and causative of the pathogenic features in DM1 [1,45-49]. Here, we report that reduction in the level of expanded DMPK transcripts is coupled to a partial normalization of the myogenic transcriptome, but that this is not overtly associated with correction of representative splice aberrations. Interestingly, in a GEP study in mice, other researchers found that dysregulation of gene expression caused by expanded (CUG)n repeats is not completely dependent on Mbnl loss, which is thought to be responsible for more than 80% of DM1 alternative splice changes [50]. Based on our preliminary findings and these literature observations (realizing that we still have to do a much more in depth study of effects on alternative splicing) it is tentative to speculate that these RNA processing alterations could be downstream of primary repeat effects. Distinction between primary and secondary effects in pathogenesis has also been suggested by others [51]. Part of the splicing changes observed in DM1 may therefore not be causal in development of the DM1 phenotype. Since most of these changes represent a switch from adult to embryonic splice forms [52,53], perhaps they can be explained as an repeat-mediated effect of transition of muscle cells to a less differentiated state [54].
ACKNOWLEDGEMENTS

We thank Jeroen van de Giessen (BioMarin Nederland BV) for conducting initial attempts in the processing of RNA samples for SAGE; Henk Buermans (Leiden Genome Technology Center) for providing information about the RNA-Seq procedure. We thank members of the Department of Cell Biology and BioMarin Nederland for meaningful discussions.
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Transcriptome analysis of cells treated with (CAG)7

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Chapter 4

CHAPTER 5

CELL MEMBRANE INTEGRITY IN MYOTONIC DYSTROPHY TYPE 1: IMPLICATIONS FOR THERAPY

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ABSTRACT

Myotonic Dystrophy type 1 (DM1) is a multisystemic disease caused by toxic RNA from a DMPK gene carrying an expanded (CTG•CAG)n repeat. Promising strategies for treatment of DM1 patients are currently being tested. These include antisense oligonucleotides and drugs for elimination of expanded RNA or prevention of aberrant binding to RNP proteins. A significant hurdle for preclinical development along these lines is efficient systemic delivery of compounds across endothelial and target cell membranes. It has been reported that DM1 patients show elevated levels of markers of muscle damage or loss of sarcolemmal integrity in their serum and that splicing of dystrophin, an essential protein for muscle membrane structure, is abnormal. Therefore, we studied cell membrane integrity in DM1 mouse models commonly used for preclinical testing. We found that membranes in skeletal muscle, heart and brain were impermeable to Evans Blue Dye. Creatine kinase levels in serum were similar to those in wild type mice and expression of dystrophin protein was unaffected. Also in patient muscle biopsies cell surface expression of dystrophin was normal and calcium-positive fibers, indicating elevated intracellular calcium levels, were only rarely seen. Combined, our findings indicate that cells in DM1 tissues do not display compromised membrane integrity. Hence, the cell membrane is a barrier that must be overcome in future work towards effective drug delivery in DM1 therapy.
INTRODUCTION

Myotonic Dystrophy type 1 (DM1) is the most common form of muscular dystrophy in adults. Patients with this disease carry an unstable (CTG)n repeat in the 3' UTR of the DMPK gene, the length of which correlates with disease severity [1,2]. DM1's molecular pathogenesis is complex. Firstly, mutant DMPK RNAs with a long (CUG)n repeat are retained in the cell nucleus, where they abnormally bind transcription and splicing factors, resulting in aberrant protein production and different downstream cellular effects [3]. Secondly, antisense transcripts from the mutant DM1 locus, which carry an expanded (CAG)n repeat, may contribute to the imbalance in proteostasis in DM1 by the production of homopolymeric proteins via a process called RAN translation [4]. Combined, these effects of (CTG•CAG)n expansion are thought to compromise functional development and cause wasting of skeletal muscle (myotonia and muscle weakness), heart (arrhythmia) and brain (mental retardation).

No cure for DM1 is available yet, but strategies for molecular therapy based on antisense RNA, siRNA or oligonucleotides (AONs) [5-11], compounds that inhibit aberrant (CUG)n RNA-protein interactions [12-14] or site-specific RNA endonucleases that target (CUG)n repeats [15] are currently under development. As essentially all these strategies require intracellular delivery of the therapeutic agents (oligonucleotides, high molecular weight organic compounds or proteins), sophisticated approaches may be needed to promote uptake across biological membranes and reach effective tissue concentrations [e.g. 16].

Typically, efficacy of drug uptake into cells is dominantly controlled by molecular characteristics of the cargo itself and by properties of the membranes. Functional changes in cell membranes are central in the pathogenesis of many diseases [17]. For example, membrane permeability can be altered by aberrant protein-membrane interactions, presence of aggregative proteins [18] or lack of integral membrane proteins, i.e. as seen with dystrophin in patients with Duchenne muscular dystrophy (DMD) [19].

Despite its importance as parameter for efficacy of drug delivery, not much is known about membrane integrity in tissues of DM1 patients. Abnormal red blood cell membranes in DM1 patients were noticed in the past [20-22]. Pathological features in skeletal muscles include internal nuclei, ring fibers, sarcoplasmic masses, type-I fiber predominance and atrophy, fibrosis and fatty infiltration, and a greatly increased number of intrafusal muscle fibers [23]. Potentially related to the myopathy, DM1 patients may show mildly elevated levels of markers of muscle damage in serum [24], with a possible impact of exercise regimen on these parameters [25]. Finally, aberrant splicing of dystrophin in DM1 patients has been reported [26], with as yet unknown effects on muscle membrane function. All data combined suggest that a certain level of membrane leakiness cannot be excluded in the highly complex DM1 phenotype.

Here, we investigated the possible involvement of membrane permeability in the context of AON-mediated treatment for DM1 using mouse models that replicate DM1 characteristics, i.e. in HSA\textsuperscript{LR} mice [27] and in DM500 [28,29] and DMSXL mice [30], both descendants from the DM300-328 line [31] (Table 1). Better understanding of cell membrane properties in these animal models will support preclinical development of effective therapeutic strategies in DM1 patients. We report on membrane integrity and related membrane characteristics in...
these models, in comparison to findings in wild type (WT) mice and mdx mice, a DMD mouse model with leaky muscle membranes [32,33]. For comparison, these parameters were also studied in muscle biopsies from DM1 patients. Our study combining mouse and human samples demonstrates that cells in DM1 tissues most likely have a functional membrane. Future therapeutic studies in DM1 mouse models may therefore benefit from advanced targeting strategies for effective therapeutics for DM1.

### MATERIALS AND METHODS
**Ethics Statement**

Animal experiments were approved by the Animal Ethics Committee of Radboud University (Permit Number: RU-DEC 2012-102).

Human control #1 quadriceps muscle tissue was obtained from the VU University Medical Center (VUmc, Amsterdam, The Netherlands). The use of post-mortem material was approved by the VUmc research committee (project 2011-67), where relatives have given explicit prior written consent that tissue taken at autopsy can be used for research, after completion of the diagnostic process and informed consents were approved by institutional review boards.

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Transgene/Mutation</th>
<th>Promoter/Expression</th>
<th>Phenotype/Symptoms</th>
<th>Original references</th>
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<td>Human DM1 locus with ~500 CTG triplets</td>
<td>Human DMPK promoter/ all DM1 related tissues (e.g., skeletal muscle, heart, CNS)</td>
<td>Homozygous mice: myopathy, reduced muscle strength, myotonia (generally very mild phenotype)</td>
<td>[28,29]</td>
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<td>Human DM1 locus with ~1600 CTG triplets</td>
<td>Human DMPK promoter/ all DM1 related tissues (e.g., skeletal muscle, heart, CNS)</td>
<td>Homozygous mice: myopathy, reduced muscle strength, myotonia, reduced body size (more severe phenotype than DM500 mice)</td>
<td>[30]</td>
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<td>HSA LR</td>
<td>Human α-actin gene with ~250 CTG triplets</td>
<td>Human α-actin promoter/ skeletal muscle only</td>
<td>Homozygous mice: strong myotonia, myopathy, no muscle weakness</td>
<td>[27]</td>
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<td>Mdx</td>
<td>Spontaneous point mutation in dystrophin gene</td>
<td>Ubiquitous (e.g., skeletal muscle, heart, CNS, retina)</td>
<td>Hemizygous mice: muscle degeneration and atrophy, skeletal muscle fibrosis and necrosis</td>
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<td>WT</td>
<td>No transgene (genetic background &gt;90% C57BL/6)</td>
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Human control #2 and #3 quadriceps muscle tissues were obtained from a commercial tissue bank (Cambridge Bioscience, Cambridge, UK; www.bioscience.co.uk). The bank provides frozen human tissue samples that have been collected from pre-consented post-mortem donors in the UK.

Quadriceps muscle biopsies from the six DM1 patients were taken by B. van Engelen in a regular diagnostic procedure, performed routinely in the neuromuscular clinic. Oral informed consent was received from all patients, documented in the patients’ charts, to perform the procedure and to use the muscle material for research purposes.

A quadriceps biopsy from a DMD patient was authorized by Dr. M. Tulinius (University of Göteborg, Sweden), with written informed consent for use in research from the patient’s parents, with approval by the Local Ethics Committee in Göteborg.

All biopsies were anonymized before they were handed over for research.

**Human material**

All information regarding human muscle samples used in this paper is summarized in Table 2.

**Mice**

DM500, DMSXL, HSA\[^{LR}\] and WT mice (similar background as DM500/DMSXL strains, i.e. >90% C57BL/6) were bred under SPF conditions in the Central Animal Laboratory in Nijmegen. Female homozygous and male hemizygous mdx mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). For the DM500, DMSXL and HSA\[^{LR}\] lines homozygous mice were used in experiments. From each mouse strain animals of different ages were used (2-6 months for EBD experiments, average 3.5 months; 2-3 months for CK measurements, average 2.2 months) to avoid bias with regard to possible developmental changes. Male and female mice were used to exclude gender-specific effects.

**Exercise regimen in treadmill**

A five-lanes treadmill (Panlab/Harvard Apparatus, Holliston, MA, USA) with air-puff control system and 0° slope inclination was used in the exercise regimen (adapted from [34]). Mice were settled with the treadmill belt stationary for 2 minutes, followed by a warm-up period of 8 minutes at a speed of 8 m/minute, immediately followed by the main exercise regimen of 30 minutes at 12 m/minute. Five mice participated in each exercise session (one animal of each strain). All mice performed well in the exercise regimen, except in one session, in which one DMSXL mouse and one mdx mouse stopped running after 20 minutes. In that case the treadmill was stopped and a rest period of 2 minutes was given to all mice. After that, the exercise was continued for 10 minutes without further difficulties.

**Mouse blood collection and serum CK assay**

Blood was collected before and after exercise via a tail cut (~100 µl/mouse). Samples were incubated for 1 hour at room temperature to allow clotting and were then centrifuged
at 12,000g for 10 minutes at 4°C. Serum was collected and snap frozen in liquid nitrogen before storage at -80°C. Serum samples were diluted 1:10 in 0.9% NaCl prior to analysis. CK activity was determined using the ARCHITECT system (Abbott Laboratories, Abbott Park, IL, USA) based on the IFCC method for the measurement of catalytic activity of CK [35] at the Department of Laboratory Medicine, Radboudumc.

**EBD injection and tissue isolation**

Evans Blue Dye (Sigma-Aldrich, St. Louis, MO, USA), 10 µg/µl in physiological saline (0.15 M NaCl, 10 mM phosphate buffer, pH 7.4), sterilized by passage through a 0.2 µm pore size membrane filter was injected intravenously through the tail vein 30 minutes after exercise (50 µl/10 g body weight). Mice were sacrificed by cervical dislocation 24 hours later. Tissues were immediately harvested and frozen in isopentane pre-chilled in liquid nitrogen.

**Table 2. Summary of information on patients and human controls and results from this study**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gender</th>
<th>DM1 phenotype</th>
<th>Estimated (CTG)n length in blood</th>
<th>CK value, in U/L (age of sampling)</th>
<th>Age at muscle biopsy, in years</th>
<th>Estimated (CTG)n length in muscle biopsy (see Fig. S6)</th>
<th>Dystrophin expression in muscle membrane, in a.u. (mean ± CI)</th>
<th>Calcium-positive fibers in muscle biopsy</th>
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<tbody>
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<td>n.a.</td>
<td>n.a.</td>
<td>n.k.</td>
<td>62</td>
<td>n.a.</td>
<td>146 ± 3</td>
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<td>n.a.</td>
<td>n.a.</td>
<td>n.k.</td>
<td>83</td>
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<td>Control #3</td>
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<td>n.a.</td>
<td>n.a.</td>
<td>n.k.</td>
<td>48</td>
<td>~100-200</td>
<td>103 ± 4</td>
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<td>Adult</td>
<td>&gt;200</td>
<td>670 (30), 1182 (32.6), 173 (32.9)</td>
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<td>~100-300</td>
<td>121 ± 5</td>
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</tr>
<tr>
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<td>n.k.</td>
<td>173 (n.k.)</td>
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<td>86 ± 6</td>
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<td>735 (n.k.), 1198 (n.k.)</td>
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<td>DM1 #4</td>
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<td>~80-500</td>
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<td>DMD</td>
<td>Male</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.k.</td>
<td>5</td>
<td>n.a.</td>
<td>7 ± 0.3</td>
<td>Abundant</td>
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n.a.: not applicable; n.k.: not known; n.d.: not determined
**Tissue cryosectioning**

10 µm tissue sections were prepared from a representative middle part of each of the six tissues examined according to standard procedures using a Microm HM 500 Cryostat (Adamas Instrumenten B.V., Rhenen, The Netherlands), dried and stored at -80°C until further use.

**EBD quantification**

For EBD imaging, sections were dried at room temperature, dipped in acetone, xylene and mounted in DPX mounting solution (Fisher Scientific, Loughborough, UK). Images were acquired by red autofluorescence in a Leica DMI6000B automated high-content microscope (Leica Microsystems, Wetzlar, Germany) using the TRITC filter. The percentage EBD-positive area was calculated by dividing the EBD-positive area by the total area of the section using ImageJ software.

**Immunohistochemistry**

Mouse muscle cryosections were dried at room temperature and incubated for 1 hour in PBS with 0.05% Tween-20 and 5% horse serum, followed by three washing steps in PBS for 5 minutes. Rabbit anti-dystrophin antibody ab15277 (Abcam, Cambridge, UK) was diluted in PBS with 0.05% Tween-20 and 5% fetal bovine serum, applied to the sections and incubated for 2 hours. After washing in PBS 3 times for 5 minutes, sections were incubated with Alexa 488-conjugated goat anti-rabbit in PBS with 0.05% Tween-20 for 1 hour. Samples were rinsed in PBS and then mounted in Mowiol containing 2.5% sodium azide overnight at room temperature. Images were acquired by epifluorescence using the Leica microscope.

Immunostaining and quantification of dystrophin in human samples was done as described [36]. In short, muscle cryosections were dried at room temperature and fixed in acetone for 1 minute, rinsed and washed with PBS for 5 minutes and blocked for 1 hour in PBS with 0.05% Tween-20 and 5% horse serum, followed by another rinsing and washing step in PBS. Rabbit anti-dystrophin ab15277 or IgG isotype antibody (used as control) were diluted in PBS with 0.05% Tween-20 and 5% fetal bovine serum and applied to the sections for 2 hours, followed by 1 hour incubation with ab15277 and mouse anti-spectrin (Novocastra, Newcastle, UK) combined. After rinsing and washing in PBS twice for 5 minutes, sections were incubated with Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-mouse in PBS with 0.05% Tween-20 for 1 hour. Samples were rinsed and washed twice for 5 minutes in PBS and then mounted using Vectashield (Vector Labs, Burlingame, CA, USA). Images were acquired using the Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany). The healthy control muscle section displaying the highest dystrophin intensity was used to adjust the confocal settings for the Alexa 488 and 594 channel. These parameters were maintained for all other sections. To minimize variations in laser intensity due to changes in lamp temperature, the laser intensity was kept constant by performing a calibration using a mirror slide. Four to five images per patient or control were acquired, which were subsequently processed using Definiens Architect software (Definiens, Munich, Germany). The software used spectrin signal to locate the membrane of muscle
fibers and to define the region of interest. The operator manually excluded areas that were not correctly identified by the program. Dystrophin intensity was calculated in the region of interest, giving a representation of DMD expression per muscle fiber.

**Calcium staining**
Muscle sections were dried at room temperature and stained for 5 minutes in 1% Alizarin Red S (Sigma-Aldrich, St. Louis, MO, USA) in distilled water adjusted to pH 5.4 with ammonium hydroxide, as recommended [37]. Samples were dehydrated in acetone (20 dips), incubated in acetone-xylene (1:1) solution (20 dips) and cleared in xylene prior to mounting in DPX mounting solution (Fisher Scientific, Loughborough, UK). Muscle slides were left to dry overnight at room temperature and then stored at 4°C. Images were acquired in the Leica microscope with a TX2 cube and a DFC480 color camera (Leica Microsystems, Wetzlar, Germany).

**CTG)n length determination by heat pulse extension PCR**
Genomic DNA was isolated from muscle sections following standard procedures. To amplify expanded (CTG)n repeats a heat pulse extension PCR protocol was used [38], that allows the generation of DMPK (CTG)n amplicons of up to 1750 CTG repeats. 40 ng DNA was used in a PCR mixture containing 2.25 M betaine (Fluka, Sigma-Aldrich, Germany), 0.2 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 1.33 units DyNAzyme EXT DNA Polymerase (Thermo Scientific, Waltham, MA, USA) and 250 nM of each forward (5´-GCCAGTTCCAAACGGCTCAGGGTGTC-3´) and reverse (5´- ACGCTCCCCAGACGAGGGTGTCATGC-3´) primers (Biolegio BV, Nijmegen, the Netherlands). Cycling conditions were kept as described [38], except that an annealing temperature of 66°C followed by ramping to 83°C (0.9 °C/sec) was used. PCR was performed in a DNA engine Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The size of the expected DMPK amplicon was 324 bp long excluding the (CTG)n repeat.

PCR products were run on a 1% agarose gel and transferred to a Hybond-XL nylon membrane (Amersham, GE Healthcare). The blot was incubated with a ^32^P-end-labeled (CAG)9 probe and after washing exposed to a phosphor imager screen (Kodak, Rochester, NY, USA) for 3 hours. Signal was developed using a Personal FX Phosphor Imager (Bio-Rad).

**Statistics**
To compare values of serum CK and EBD-positive fibers between groups, we used a one-way ANOVA test. We applied a paired Student’s t-test to assess whether serum CK activity was significantly different before and after the exercise regimen. All values in graphs are presented as mean ± SEM (unless indicated otherwise). Differences between groups were considered significant when P<0.05: *, P<0.05; **, P<0.01; ***, P<0.001. Statistical analyses were performed with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).
RESULTS
For our study of cell membrane integrity we compared WT mice with DM500, DMSXL and HSA<sup>L</sup> mice, three transgenic models frequently used in preclinical DM1 studies [39] (Table 1). For an overt DM1 muscle phenotype, all three models were bred to homozygosity. Mdx mice, a well-established model for DMD, were included as positive control, as these demonstrate increased muscle fiber membrane permeability. To minimize possible effects of differences in physical activity between mice and to stimulate muscle contraction, mice were subjected to a 30 minute exercise protocol on a motorized treadmill. Before and after exercise several biomarkers for cell membrane integrity were measured.

Creatine kinase activity in mouse serum
Creatine kinase (CK) is released in the circulation upon muscle damage [40] or because of disease-induced loss of membrane integrity. Dystrophin-deficient mdx mice, for example, show an increased basal serum CK level compared to control, which rises significantly after exercise [34]. Under basal conditions, the three DM1 models showed similar serum CK values as WT controls (Fig. 1A). In contrast, the corresponding levels in mdx mice were indeed around 20-fold higher. After exercise, serum CK levels increased only around 2-fold in WT, DM500 and HSA<sup>L</sup> animals (Fig. 1B), whereas a 40-fold increase was observed in mdx mice. Markedly enhanced sensitivity of DMSXL mice to exercise was detected, compared to the other DM1 models and WT mice (6-fold increase in CK level; Fig. 1). This effect was probably due to their more severe muscle weakness phenotype, leading to extra muscle wasting necessary to complete the exercise regime, rather than to intrinsic muscle membrane permeability of the model. Nevertheless, neither basal CK levels nor levels after exercise in DMSXL mice were statistically different from those in WT mice.

Evans Blue Dye uptake in mouse cells in vivo
To evaluate vasculature of living animals and integrity of cell membranes in vivo after exercise, we used cell membrane-impermeable Evans Blue Dye (EBD) as tracer [41,42]. EBD binds to albumin in the bloodstream, leaks into muscle fibers that are damaged and can be observed microscopically by its red autofluorescence. DM1 mice did not show permeability to EBD in any of the skeletal muscles investigated (quadriceps, gastrocnemius, tibialis anterior, diaphragm), similar to WT controls (Fig. 2A,B; Supporting Figs S1-3). As expected [42], relatively large, damaged EBD-positive areas were present in mdx muscle. Only once, very few isolated positive fibers were found in a quadriceps muscle of a DMSXL mouse (data not shown). We did not find positive fibers in heart in any of the mice examined (Supporting Fig. S4), although it has been reported that ~50% of mdx mice show EBD-positive fibers in this tissue [41]. Finally, no EBD uptake was observed in brain in any of the mice examined (Supporting Fig. S5).

Dystrophin expression
Deficiency of dystrophin, a protein involved in muscle membrane structure and flexibility, leads to progressive muscular dystrophy and degeneration in DMD patients and mdx
mice [32]. Also in DM1 patients, abnormal production of dystrophin isoforms has been demonstrated [26]. We verified dystrophin protein expression by immunohistochemistry in muscles of exercised DM1 animals and found that the staining pattern was indistinguishable from that in WT mice in all tissues analyzed (Fig. 2C and Supporting Figs S1-4). As expected, apart from few so-called revertant fibers [43], no dystrophin staining was detected in mdx mice.

**Cell membrane integrity in DM1 patients**

To evaluate our findings obtained in DM1 mouse models, we investigated muscle membrane integrity in human quadriceps muscle. We used biopsies from three healthy controls, six DM1 patients and one DMD patient. (CTG)n repeat lengths in the DM1 muscle biopsies...
Figure 2. Membrane integrity analysis in mouse quadriceps muscle. (A) Representative images of quadriceps sections from DM1 mice and controls after injection with EBD following exercise. One WT mouse was not injected to appreciate autofluorescent background signal (No EBD). Areas with EBD-positive fibers were regularly seen in mdx samples (arrowheads), but never in WT nor DM1 model samples. Scale bars indicate 250 µm. (B) Quantification of the EBD-positive area compared to total muscle section (n=4 per group). (C) Representative images of quadriceps sections stained for dystrophin. Staining intensity and pattern observed in WT animals were very similar to those observed in DM500, DMSXL and HSA<sub>LR</sub> mice. Right panels show high magnifications of insets to appreciate dystrophin staining. As expected, essentially no signal was detected in mdx mice. Scale bars indicate 150 µm.
ranged between ~80 and >500, as measured by heat pulse extension PCR [38] (Supporting Fig. S6; Table 2). For some of these patients slightly elevated CK levels had been measured in the past (Table 2).

As an alternative method to the EBD injections done in mice, we performed Alizarin Red staining on human muscle sections to detect elevated intracellular calcium levels or deposits, suggestive of membrane abnormalities [37]. Calcium-positive muscle fibers were indeed abundant in DMD muscle (Fig. 3A, Table 2). However, no or only few calcium-positive fibers were observed in controls and DM1 patients.

Next, we performed immunostaining of dystrophin in serial muscle sections from the same patients and controls. Spectrin immunostaining was used to visualize the membrane of muscle fibers. In all healthy control and DM1 biopsies overt cell membrane expression of dystrophin was observed, whereas essentially no dystrophin protein was detected in the DMD sample (Fig. 3B). We quantified the amount of dystrophin using a novel method for immunofluorescence quantification [36]. Dystrophin expression in DM1 samples turned out to be similar to that in control samples (Fig. 3C, Table 2). As expected, dystrophin level in the DMD sample was extremely low.

| DISCUSSION |

Efficient systemic cellular uptake of therapeutics is an important determinant for the success of future clinical trials in DM1, because all known molecular targets, e.g., expanded (CUG)n/(CAG)n transcripts or CELF1 and MBNL1 proteins that bind to these repeat-containing RNAs [44] are located intracellularly. The effective concentration of any DM1 drug will therefore depend on its chemical and pharmacokinetic characteristics and the ability to pass plasma membranes of endothelial cells and target cells in muscle, heart or brain of patients. From research on DMD, we have learned that the disease-related loss of dystrophin alters membranes of muscle and brain cells, leading to increased permeability for oligonucleotides and other large molecules [19].

In this study, in comparison to the situation in DMD, we examined cell membrane integrity in DM1 mouse models and in patient muscle biopsies. Our findings on human material suggest that cell membranes in DM1 patients are intact. Expression and localization of dystrophin was normal. We therefore assume that sufficient functionally active protein was present to support membrane function, in spite of an abnormal dystrophin splice mode that affects the protein’s c-terminus responsible for interaction with the sarcolemma [26]. Furthermore, in concordance with previous studies [37], calcium deposits, indicative of membrane damage, were rare or absent in DM1 muscle biopsies tested.

In a group of moderately affected, ambulatory DM1 patients one in four male patients and one in two female patients showed mildly elevated basal CK values in serum [24]. Fluctuating, slightly elevated CK levels were also measured in our small cohort of DM1 patients (Table 2). These elevated CK values could be related to a mild membrane defect but, more likely, they are related to myopathy and associated with tissue loss in these patients. More extensive clinical studies in a much larger cohort of individuals are necessary to relate CK values in serum to known variables of DM1 manifestation – e.g. (CTG•CAG)n-repeat length, somatic
Figure 3. Membrane integrity analysis in DM1 patient biopsies. (A) Calcium staining using Alizarin Red S. No or only few calcium-positive fibers (arrowheads), indicative of abnormal elevated calcium level, were found in DM1 and control biopsies. Calcium deposits were abundant in the DMD sample. DMD muscle sections showed fatty tissue infiltration (asterisks) and fibrosis (stars), which were also detected in some of the DM1 patient biopsies. Scale bars indicate 100 μm. Results from only one of the three controls and three of the six examined DM1 patients are shown. All data are summarized in Table 2. (B) Dystrophin immunostaining. In contrast to the DMD patient biopsy, expression and localization of dystrophin in DM1 and control quadriceps muscles were normal. Spectrin staining was included as a cell membrane reference. Scale bars indicate 100 μm. (C) Quantification of dystrophin expression. Data is represented as mean intensity per fiber (n>50). Error bars indicate 95% confidence interval (CI). Data are summarized in Table 2.
repeat expansion rate, disease onset and severity, and exercise and life-style habits – before we can draw further conclusions.

The main focus of our study was to obtain better understanding of cell membrane integrity in transgenic DM1 mouse models currently in use for testing new therapies [44]. From a series of observations in models that faithfully replicate most important characteristics of disease in DM1 [39], we conclude that cell membrane function is not affected by presence of repeat RNA. Firstly, we found that serum CK levels in all three models are normal. Even after intense exercise, only a two- to six-fold increase was measured, similar to wild type control animals. Secondly, dystrophin immune-staining was comparable to that in WT mice. Thirdly, we did not observe permeability to EBD in any of the tissues analyzed. With respect to the HSA<sup>LR</sup> model, our microscopy findings match those presented in an earlier report, where EBD presence was measured in whole muscle lysates by spectrophotometry, using a protocol without exercise regimen [9].

It should be noted that we cannot exclude the possibility that cell membrane integrity in DM1 mice might deteriorate during aging. This aspect was not part of our study and we chose the age group of 2-6 month-old mice, since mice of that age are usually included in therapeutic studies for DM1. For the DMSXL strain in particular, it has been shown that the strongest symptoms are indeed seen in rather young mice [30].

Most of the treatments for DM1 that are currently under development involve the use of relatively large compounds that neutralize expression of expanded DMPK transcripts or block binding to MBNL1 [44,45]. For these approaches to become effective, active compounds must reach the cell nucleus, i.e., the cellular compartment where most of the toxic (CUG)<sub>n</sub> RNA is located. Since we find no evidence for membrane alterations or increased membrane permeability in the transgenic DM1 mice, we assume that drugs that were administered systemically and showed efficacy in preclinical studies must have reached their destination via naturally existing cellular uptake and routing mechanisms either independently [9, Mulders et al. unpublished,12] or promoted by advanced targeting moieties [8, Mulders et al., unpublished]. Based on our findings, we consider it unlikely that the disease state had a major effect on drug fate in these studies, although we do not know whether carrier-mediated transport, receptor-mediated endocytosis or any other transport route was involved [46,47].

In sum, we conclude that cell membranes of DM500, DMSXL and HSA<sup>LR</sup> mice are intact and have normal physical stress resistance and properties for chemical passage. Our observations in muscle biopsies from DM1 patients corroborate this picture. Further study is now necessary to know how compounds that have been shown to bind or degrade (CUG)<sub>n</sub> RNA are internalized by myofibers or other cell types. Future therapeutic studies in DM1 mouse models may benefit from advanced targeting strategies for effective therapeutics for DM1, given the fact that cell membranes are probably intact in patients. As there is no need to concentrate these studies on DM1-affected tissues only, this opens up prospects for the simultaneous development of therapy for other neurodegenerative disorders.
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<td>We thank A. Janson, J. Testerink and B. Aguilera (Prosensa) for technical support and discussions; D. Reijnen (Central Animal Laboratory) for help with mouse injections; G. Martens, J. Visser and K. Klemann (Dept. Molecular Animal Physiology and Dept. Neurology) for providing us with the treadmill; J. Verhagen (Dept. Laboratory Medicine) for CK analysis; and B. Küsters (Dept. Pathology) for help with human biopsies. We thank members of the Department of Cell Biology for discussions.</td>
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AGB, SB, JCTvD and SAMM are employees (which includes contribution to patent applications and participation in stock-option plans) of Prosensa Therapeutics B.V., a company that develops RNA therapeutics for Duchenne muscular dystrophy and other neuromuscular disorders. This does not alter the authors’ adherence to PLOS ONE policies on sharing data and materials (biopsies cannot be shared owing to availability and clinical consents).
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SUPPLEMENTARY MATERIALS

Figure S1. Membrane integrity analysis of gastrocnemius muscle. (A) Representative images of gastrocnemius sections from DM1 mice and controls after injection with EBD after exercise. Scale bars indicate 200 µm. One WT mouse was not injected to appreciate autofluorescent background signal (No EBD). Areas with EBD-positive fibers were regularly seen in mdx samples (arrowheads) but never in WT nor DM1 model samples. (B) Quantification of percentage of EBD-positive area compared to total muscle section (n=4 per group). (C) Representative images of gastrocnemius sections stained for dystrophin. Scale bars indicate 150 µm. Staining intensity and pattern observed in WT animals were very similar to those observed in DM500, DMSXL and HSA<sup>L</sup>R mice. As expected, essentially no signal was detected in mdx mice.
Figure S2. Membrane integrity analysis of tibialis anterior muscle. (A) Representative images of tibialis anterior sections from DM1 mice and controls after injection with EBD after exercise. Scale bars indicate 200 µm. One WT mouse was not injected to appreciate autofluorescent background signal (No EBD). Areas with EBD-positive fibers were regularly seen in mdx samples (arrowheads) but never in WT nor DM1 model samples. (B) Quantification of percentage of EBD-positive area compared to total muscle section (n=4 per group). (C) Representative images of tibialis anterior sections stained for dystrophin. Scale bars indicate 100 µm. Staining intensity and pattern observed in WT animals were very similar to those observed in DM500, DMSXL and HSA LR mice. As expected, essentially no signal was detected in mdx mice.
Figure S3. Membrane integrity analysis of diaphragm muscle. (A) Representative images of diaphragm sections from DM1 mice and controls after injection with EBD after exercise. Scale bars indicate 100 µm. One WT mouse was not injected to appreciate autofluorescent background signal (No EBD). Areas with EBD-positive fibers were regularly seen in mdx samples (arrowhead) but never in WT nor DM1 model samples. (B) Quantification of percentage of EBD-positive area compared to total muscle section (n=4 per group). (C) Representative images of diaphragm sections stained for dystrophin. Scale bars indicate 100 µm. Staining intensity and pattern observed in WT animals were very similar to those observed in DM500, DMSXL and HSA\textsuperscript{LR} mice. As expected, essentially no signal was detected in mdx mice.
Figure S4. Membrane integrity analysis of heart muscle. (A) Representative images of heart sections from DM1 mice and controls after injection with EBD after exercise. Scale bars indicate 100 µm. One WT mouse was not injected to appreciate autofluorescent background signal (No EBD). (B) Representative images of heart sections stained for dystrophin. Scale bars indicate 100 µm. Staining intensity and pattern observed in WT animals were very similar to those observed in DM500, DMSXL and HSA LR mice. As expected, essentially no signal was detected in mdx mice.
Figure S5. Absence of Evans Blue Dye in brain. Representative images of mesencephalon (midbrain) from total brain sagittal sections of DM1 mice and controls after injection with EBD after exercise. Scale bars indicate 150 µm. One WT mouse was not injected to appreciate autofluorescent background signal (No EBD). No EBD signal was found in any other brain region either.
Figure S6. (CTG)n repeat length determination in human muscle biopsies. Genomic DNA isolated from human muscle biopsies was used in a heat pulse extension PCR protocol to amplify the (CTG)n repeat of DMPK genes. Control sample represents genomic DNA from a healthy individual carrying a (CTG)5 and a (CTG)12 repeat. The smears in patient samples demonstrate somatic instability. The size marker at the left indicates the estimated number of CTG triplets. Note that lane 1 and 2 were exposed longer for clarity.
CHAPTER

6

INTRACELLULAR DISTRIBUTION
AND NUCLEAR ACTIVITY OF ANTISENSE
OLIGONUCLEOTIDES AFTER UNASSISTED
UPTAKE IN MYOBLASTS AND
DIFFERENTIATED MYOTUBES IN VITRO

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Clinical efficacy of antisense oligonucleotides (AONs) for the treatment of neuromuscular disorders depends on efficient cellular uptake and proper intracellular routing to the target. Selection of AONs with highest in vitro efficiencies is usually based on chemical or physical methods for forced cellular delivery. Since these methods largely bypass existing natural mechanisms for membrane passage and intracellular trafficking, spontaneous uptake and distribution of AONs in cells are still poorly understood. Here, we report on unassisted uptake of naked AONs, so-called gymnosis, in muscle cells in culture. We found that gymnosis works similarly well for proliferating myoblasts as for terminally differentiated myotubes. Cell biological analyses combined with microscopy imaging showed that a phosphorothioate backbone promotes efficient gymnosis, that uptake is clathrin-mediated and mainly results in endosomal-lysosomal accumulation. Nuclear localization occurred at low level, but the gymnotically delivered AONs effectively modulated expression of their nuclear RNA targets. Chloroquine treatment after gymnotic delivery helped to increase nuclear AON levels. In sum, we demonstrate that gymnosis is feasible in proliferating and non-proliferating muscle cells and confirm the relevance of AON chemistry for uptake and intracellular trafficking with this method, which provides a useful means for bio-activity screening of AONs in vitro.
Free uptake of oligos in muscle cells in vitro

INTRODUCTION

Antisense oligonucleotide (AON) therapeutics involve a broad range of approaches to adjust levels of RNA targets via effects on posttranscriptional processes [1]. AONs are in (pre)clinical development for many diseases, including cancer, inflammatory conditions, cardiovascular disease and neurodegenerative and neuromuscular disorders. Clinical efficacy of AONs largely depends on biodistribution to and uptake by target cells and routing to the desired intracellular location (nucleus or cytoplasm). Although this is true for any disease for which AON therapy is an option, it is especially relevant for degenerative muscle disorders. In this class of diseases, muscle tissue throughout the entire body can be affected, requiring a therapeutic effect in up to 40% of total body mass. Examples of multisystemic neuromuscular disorders for which AON therapy is currently under development are spinal muscular atrophy [2], Pompe disease [3], iron-sulphur cluster deficiency myopathy [4], Duchenne muscular dystrophy (DMD; [5]) and myotonic dystrophy type 1 (DM1; [6,7]).

Here we focus on DMD and DM1, both muscular dystrophies characterized by typically early and highly variable onset, respectively, with progressive general muscle dysfunction [8]. DMD, the most common lethal chromosome X-linked disease in man, is caused by mutations in the dystrophin (DMD) gene creating a premature stop codon that abrogates synthesis of functional dystrophin protein [9]. As dystrophin is a structural protein responsible for connecting the cytoskeleton of muscle fibers to the extracellular basal lamina, its absence affects membrane stability of myofibers, eventually leading to cell death and replacement by connective tissue [10]. Myotonic dystrophy type 1 (DM1), the most common muscular dystrophy in adults [11], has a different etiology. Expansion of an unstable (CTG)n repeat in the 3’ UTR of the DMPK gene is responsible for a highly variable multisystemic phenotype [11,12]. When the number of (CTG)n triplets in the repeat exceeds a certain threshold, expanded DMPK (CUG)n transcripts are produced that accumulate in the cell nucleus and aberrantly bind a variety of ribonucleoprotein factors. Sequestration of these factors causes RNA processing abnormalities of other mRNAs and miRNAs resulting in a broad spectrum of toxic gain-of-function effects [13].

Therapeutic correction at the RNA level by AON-mediated exon skipping of mutant dystrophin pre-mRNA is a promising disease-modifying treatment option for DMD [5]. Similarly, for DM1, proof-of-concept studies have demonstrated that gene silencing via AON-mediated breakdown and prevention of nuclear accumulation of expanded DMPK (CUG)n mRNA may become a useful therapeutic modality in the future [6,7,14-18]. Notably, although the therapeutic strategies for DMD and DM1 are mechanistically distinct, they share an important aspect: exon skipping of mutant DMD pre-mRNA as well as breakdown of expanded DMPK (CUG)n RNA must take place in the nucleus of target cells.

To be able to improve AON delivery to the nucleus in diseases like DM1 and DMD, it is important to study cellular uptake and intracellular distribution aspects upon treatment, including the impact that AON chemical modifications have in these processes. Preclinical selection of AON candidates is primarily based on comparative analysis of their bioactivity in cell cultures, typically using transfection or electroporation. Although these methods are useful to study bioactivity, they are less reliable predictors of cellular uptake and trafficking in vivo, since cellular barriers are artificially bypassed.
Uptake of AONs by cells in vitro without use of transfection reagent or other delivery strategy was coined gymnosis by Stein et al. in 2010 [19]. This method was based on prolonged incubation of cells with AONs dissolved in the culture medium, which progressively resulted in cellular internalization by endocytosis, via mechanisms that are currently under debate [20,21]. It is generally accepted that a small fraction of AONs is able to escape from endocytic vesicles and reach their RNA target in nucleus or cytosol, but this behavior is not well characterized either [22].

To date, unassisted uptake of AONs has been applied successfully to studies in proliferating cell types [23]. However, less is known about the applicability to non-proliferating cells, such as those that have undergone terminal differentiation. In addition, limited research has been dedicated to address how different chemical modifications of AONs influence free uptake and subsequent intracellular trafficking. Phosphorothioate moieties have been suggested to promote gymnosis, presumably through AON binding to heparin-binding proteins on the cell surface and with a role in the subsequent formation of intracellular vesicles [24]. Whether that holds for other types of AON chemistries and to what extent chemical modifications allow or influence endosomal escape, particularly in muscle cells, has remained unclear thus far.

In this study, we used a myogenic cell model derived from DM1 mice to test effects of AON chemistry and sequence on gymnosis and subsequent nuclear efficacy. Two classes of AONs were compared: (i) analogs of a CAG7 AON for degradation of expanded DMPK (CUG)n transcripts for DM1 [7,15] and (ii) variants of DMD23, an AON capable of inducing exon 23 skipping in mouse Dmd transcripts [25] (Table 1). We followed uptake and intracellular distribution of these AONs during gymnosis by means of fluorescence microscopy during myoblast proliferation and differentiation. In parallel, we measured AON activity towards nuclear DM1 and DMD targets. Facilitated uptake using polyethyleneimine (PEI) was usually included as a positive control to bypass cellular uptake and trafficking pathways. Despite the relative high subcellular AON accumulation, we found that already low levels of AONs in the nucleus may be effective. Thus, gymnosis may be a supportive method for comparative analysis of therapeutic AON candidates in (differentiated) muscle cells in vitro, that takes into account natural uptake and intracellular trafficking.

**MATERIAL AND METHODS**

**Antisense oligonucleotides**

AONs used in this study are listed in Table 1 and were synthesized by BioMarin Nederland B.V. The nucleotide sequence of CAG7 AONs is 5’-CAGCAGCAGCAGCAGCAGCAG-3’ and targets the (CUG)n repeat in expanded DMPK transcripts (PS58 [15]). The sequence of DMD23 AONs is 5’-GGCCAAACCUCGGCUUACCU-3’ and targets an exon splicing enhancer in exon 23 of mouse Dmd pre-mRNA (M23D(+02-18) [26]). Three types of chemical modification were used: 2’-O-methyl phosphorothioate (-OMePS), 2’-O-methyl phosphate (-OMe) and DNA phosphorothioate (-PS). Some AONs were conjugated at their 5’ or 3’ end with a Cy3 or FAM fluorophore or with P4, a muscle targeting peptide of sequence LGAQSNF [27].
Cy3-CAG3-ENA is a 5’-CAGCAGCAG-3’ AON with an ethylene-bridged nucleic acid (ENA) phosphate backbone, 5’ conjugated to Cy3.

**Cell culture**

DM500 myoblasts were derived from a DM1 mouse model as described [15]. DM500 myoblasts express human DMPK (CUG)500 transcripts, the disease-causing agent in myotonic dystrophy type 1 [28]. DM500 myoblasts with EGFP-stained nuclei were used for most experiments and obtained after stable transduction with a pLZRS retroviral vector expressing a EGFP-hMBNL1 fusion protein (plasmid gift from Dr. D. Brook, Nottingham). Myoblasts were cultured on 0.1% (w/v) gelatin-coated dishes in DMEM (Gibco-BRL, Gaithersburg, MD) supplemented with 20% (v/v) FCS, 50 µg/ml gentamycin, 10 U/ml IFN-γ (BD Biosciences, San Jose, CA, USA) and 2% (v/v) chicken embryo extract (Sera Laboratories International, Bolney, UK) at 33°C and 5% CO₂.

When myogenic differentiation was applied, myoblasts were seeded on Matrigel® (BD Biosciences, Breda, The Netherlands) and grown until 100% confluency. Subsequently, differentiation medium (DMEM supplemented with 5% (v/v) horse serum and 50 µg/ml gentamycin) was added and the cells were incubated at 37°C for 7-10 days. In some cases, after 3 days of differentiation, 4 µg/ml cytosine β-D-arabinofuranoside (Ara-C) (Sigma, Saint Louis, MO, USA) was added for 2 days to remove undifferentiated myoblasts.

**Gymnosis and transfection**

For gymnosis, AONs were supplemented directly in the cell culture medium. A range of concentrations (0.2-5 µM) and incubation times (4 hours to 13 days) was tested, as specified for each experiment. During proliferation, cells were seeded at 30-40% confluency and incubated with AONs in the growth medium. Every time that the medium was refreshed (every 2-3 days) or cells were passaged (before reaching confluency), fresh growth medium containing the same AON concentration was used. In experiments where myogenic differentiation was applied, cells were grown until 100% confluency and incubated with differentiation medium containing AON. Medium was refreshed every 2-3 days, containing the same AON concentration.

Polyethyleneimine (PEI)-mediated transfection of AONs was done as described [15] at a final AON concentration of 500 nM. Myoblasts and myotubes were imaged and RNA was isolated 48 hours after transfection.

**Use of endocytosis modulators**

To block clathrin-dependent endocytosis, cells were supplied with new medium containing 80 µM dynasore (Sigma-Aldrich, Saint Louis, MO, USA) at one hour prior to AON treatment. Incubation was then continued in the same medium, but containing the AON, during the entire gymnosis process (24 hours). In these experiments, DMEM supplemented with 50 µg/mL gentamycin and 10 U/ml IFN-γ, without serum, was used. Longer incubation periods were not possible due to significant cell dead and toxicity.
Table 1. AONs used in this study

<table>
<thead>
<tr>
<th>AON</th>
<th>Chemistry #</th>
<th>Sequence (5’ &gt; 3’)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG7-OMePS (PS58 [15])</td>
<td>2’-OMe PS</td>
<td>CAG CAG CAG CAG CAG CAG CAG</td>
<td>Expanded DMPK transcripts</td>
</tr>
<tr>
<td>CAG7-OMe</td>
<td>2’-OMe PO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAG7-PS [7]</td>
<td>DNA PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4-CAG7-OMePS*</td>
<td>2’-OMe PS</td>
<td>P4-CAG CAG CAG CAG CAG CAG</td>
<td></td>
</tr>
<tr>
<td>Cy3-CAG7-OMePS</td>
<td>2’-OMe PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy3-CAG7-OMe</td>
<td>2’-OMe PO</td>
<td>Cy3-CAG CAG CAG CAG CAG CAG</td>
<td></td>
</tr>
<tr>
<td>Cy3-CAG7-PS</td>
<td>DNA PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAM-CAG7-OMePS</td>
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<td>FAM-CAG CAG CAG CAG CAG CAG</td>
<td></td>
</tr>
<tr>
<td>CAG7-OMePS-Cy3</td>
<td>2’-OMe PS</td>
<td>CAG CAG CAG CAG CAG CAG-Cy3</td>
<td></td>
</tr>
<tr>
<td>P4-CAG7-OMePS-Cy3*</td>
<td>2’-OMe PS</td>
<td>P4-CAG CAG CAG CAG CAG CAG-Cy3</td>
<td></td>
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<tr>
<td>DMD23-OmePS (M23D(+02-18) [26])</td>
<td>2’-OMe PS</td>
<td>GGC CAA ACC UCG GCU UAC CU</td>
<td></td>
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<tr>
<td>DMD23-OMe</td>
<td>2’-OMe PO</td>
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<td>2’-OMe PS</td>
<td>P4-GGC CAA ACC UCG GCU UAC CU</td>
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<td>Cy3-DMD23-OMe</td>
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<tr>
<td>P4-DMD23-OmePS-Cy3*</td>
<td>2’-OMe PS</td>
<td>P4-GGC CAA ACC UCG GCU UAC CU-Cy3</td>
<td></td>
</tr>
</tbody>
</table>

# 2’-OMe = 2’-O-methyl; PS = phosphorothioate; PO = phosphate
* P4 = LGAQSNF [27]

To induce release of endosomal and lysosomal contents, cells were treated with 75 µM chloroquine (CHQ) for 4 hours at the end of the gynnosis incubation period. No signs of toxicity were observed at this CHQ concentration.

Confocal microscopy and immunochemistry
Myoblasts were grown in µ-Slide 8 well plates (ibidi®, Martinsried, Germany) or in GWSt-3522 WillCo-dish® wells (WillCo Wells B.V., Amsterdam, The Netherlands). After treatment with
Cy3-labeled AONs (Table 1), analysis was performed using a Zeiss LSM510-Meta confocal microscope. For live cell imaging, cells were put in a culture chamber that was maintained at a stable temperature of 37°C with a 5% CO₂ supply. DM500 myotubes were incubated prior to analysis with 5 µM CSFE (carboxyfluorescein succinimidyl ester; Invitrogen) for 5 minutes and with 10 µg/ml Hoechst (Life Technologies) for 30 minutes to visualize cytoplasm and nucleus, respectively. Images to evaluate AON distribution were acquired in live cells or immediately after fixation, at different time points after start of gymnosis. These approaches for image acquisition were adopted because pilot experiments revealed that standard paraformaldehyde protocols did not fix AONs permanently when the cells were kept in phosphate-buffered saline (PBS) after fixation, giving experimental variation and a false impression of their intracellular distribution (Suppl. Fig. S1). For fixation, cells were washed twice with PBS and then incubated for 15 minutes at 37°C in 0.1 M phosphate buffer (pH 7.5) with 4% (w/v) paraformaldehyde.

For visualization purposes, certain images displayed in the figures were adjusted to the maximum exposure level within the range of negative background signal. This results in overexposed areas but allows visualization of AONs in other parts of the cell where the concentration is lower, such as the nucleus. For fluorescence quantification (Fig. 1B and Fig. 6), confocal settings were calibrated below Cy3-channel saturation using the well displaying highest fluorescence signal (see Suppl. Fig. S7). Identical parameters were kept to quantify all other wells in the same microscopy session, including all treatments (n=3). Several images of each treatment (25-30 cells) were processed using ImageJ (Wayne Rasband, National Institute of Health, USA). Masks of either nuclear or cytoplasmic regions (determined by the EGFP signal) were used to select cellular areas of interest from where Cy3 intensities were measured and corrected for background (see Fig. 6).

For Lamp1 immunochemistry, cells were fixed and blocked with 4% (v/v) animal serum (corresponding to the origin of the secondary antibody) and 0.33% (v/v) Triton-X-100 in PBS for 60 minutes at room temperature. Rabbit Anti-Lamp1 primary antibody (Sigma, Saint Louis, MO, USA) was diluted in 10 mg/ml BSA and 0.33% (v/v) Triton-X-100 and applied overnight at 4°C. Samples were blocked with goat serum and incubated with Goat Anti-Rabbit alexa-647 secondary antibody (Abcam, Cambridge, UK) for one hour at room temperature.

**RNA isolation and RT-PCR analysis**

RNA was isolated from cultured cells using the Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer’s protocol. Primer sets for PCR and qPCR were designed using Primer-BLAST [29] in the NCBI database and validated in silico using OligoAnalyzer 3.1 [30] to prevent formation of hairpins and dimers during amplification (Suppl. Table S1). Resulting products were visualized on agarose gels and sequenced to verify identity.

For reverse transcription (RT), typically 0.5 µg of total RNA was subjected to cDNA synthesis using the SuperScript® first-strand synthesis system with random hexamer primers in a total volume of 20 µl.
Skipping of exon 23 in Dmd transcripts was analyzed using conventional nested PCR with primers annealing in exon 22 (forward primer) and exon 24 (reverse primer; Suppl. Table S1). Per reaction, the mix contained 10.4 µl MilliQ, 4 µl 5x Q5 Reaction Buffer, 0.2 µl Q5 High Fidelity DNA Polymerase (2U/µl) (BioLabs® Inc, Ipswich, MA, USA), 0.4 µl dNTPs (Invitrogen, Eugene, Oregon, USA), 2 µl forward primer (5 µM), 2 µl reverse primer (5 µM) (Biolegio BV, Nijmegen, The Netherlands) and 1 µl cDNA template. Thermal cycling conditions consisted of 30 seconds at 98°C followed by 34 amplification cycles of 6 seconds at 98°C, 20 seconds at 64°C (annealing) and 10 seconds at 72°C (elongation). The final elongation step was carried out for 2 minutes. Fragments were analyzed on 2% agarose gels. Images of ethidium-stained products were acquired under UV-light below pixel saturation. Quantification of signals was done using ImageJ (Wayne Rasband, National Institute of Health, USA), measuring pixel intensity and correcting for background.

For the detection of degradation of expanded DMPK transcripts real-time quantitative PCR (qPCR) was used, following a protocol previously described [7]. Samples were analyzed using the CFX96™ Real-time System (Bio-Rad, Hercules, CA, USA). A melting curve was obtained for each sample in order to confirm single product amplification. Relative mRNA levels were calculated using the ΔΔCt method [31]. Gapdh and Actb expression levels were used together for normalization.

Quantitative PCR was also used to measure mRNA levels of differentiation markers Aqp1, Myh1, Myf5 and Cav3. In this case, samples were analysed by the ViiA™ 7 Real-Time PCR System (Life Technologies, Bleiswijk, The Netherlands), by means of commercial TaqMan® assays (Applied Biosystems, Foster City, CA, USA) (Suppl. Table S1), following manufacturer’s instructions. Data was normalized using Gapdh and Hprt as reference genes. Levels of the group showing the highest expression were set to 100% using the ΔΔCt method.

Statistical analysis
We performed unpaired student’s t-tests to test whether DMPK levels after CAG7-OMePS treatment (either alone or in presence of dynasore or chloroquine) differed significantly from levels that remained after treatment with a control AON. To determine whether the average signals of Dmd skipped products were significantly different from background, we performed a t-test analysis against a zero theoretical mean. The effect of Ara-C treatment in the expression of differentiation markers was analyzed by unpaired t-tests in each gene group. To compare the effect of AONs of different chemistries on DMPK transcript levels we used a 1-way ANOVA followed by a Bonferroni’s Multiple Comparison Test. To test whether Cy3-CAG7-OMePS accumulation in time was significantly different from Cy3-CAG3-ENA behavior we used a 2-way ANOVA. In all these cases, values were considered significantly different when: *: p<0.05, **: p<0.01 or ***: p<0.001.

RESULTS
Gymnosis in proliferating myoblasts
Experiments in this study were performed in the immortal DM500 cell model [15], derived from a transgenic mouse model for DM1 [28]. DM500 myoblasts show nuclear accumulation
Free uptake of oligos in muscle cells \textit{in vitro}

of transcripts from a human \textit{DMPK} transgene bearing an expanded (CTG)\textit{S}00 repeat. The cells show normal growth and myogenic differentiation capacity. Previous studies revealed that AON transfection characteristics did not overtly differ between DM500 and normal primary mouse or human myoblasts [7,15]. Here, we mainly used a population of DM500 myoblasts that stably expressed low levels of EGFP-MBNL1, a green fusion protein with predominant nuclear location [32], for easy microscopy visualization of localization of fluorescent AONs in the nucleus.

To study gymnosis and intracellular AON distribution, we followed the behavior of Cy3-conjugated CAG7-OMePS, a 21 nt repeat AON containing a fully modified 2’-O-methyl phosphorothioate backbone (Table 1) [15]. In proliferating myoblasts, CAG7-OMePS-positive vesicular structures in the cytoplasm were present in essentially all cells after four hours of incubation with 200 nM naked oligo in the medium (Fig. 1A). The vesicular accumulation appeared more prominent after 24 hours and quantification of staining revealed that it became even more intense over the subsequent two days (Fig. 1B). Interestingly, we also noticed local accumulation of AON signal in spots in the nucleus in many, but not all cells at >24 hours of gymnosis. In addition, a diffuse nuclear staining was observed in ~1% of cells at 72 hours and ~2% after 6 days (Fig. 1C). The fraction of Cy3-positive nuclei did not further increase after longer incubation (up to 13 days) or upon use of a higher AON concentration (5 µM; data not shown).

Gymnotic uptake and distribution characteristics were entirely different from those seen with polyethylenimine-(PEI)-mediated transfection, a commonly used procedure to deliver AONs in cultured cells [33]. PEI transfection of DM500 myoblasts resulted in a predominant nuclear staining of Cy3-CAG7-OMePS in around 50% of all cells (Suppl. Fig. S2). The typical uptake and distribution behavior of naked AONs was not influenced by the type of fluorophore conjugated to the AON (Fig. 1C). Neither did the conjugation position of the fluorophore, to the 5’ or the 3’ end of the AON, have any obvious effect on AON localization (Suppl. Fig. S3).

To examine potential effects on gymnosis by the nucleotide composition of the AON we followed also the fate of Cy3-DMD23-OMePS, an AON that induces exon 23 skipping during splicing of mouse \textit{Dmd} pre-mRNA [25]. When used under the same experimental conditions, a similar localization was observed with this AON (Fig. 2A), including the predominantly vesicular and low intranuclear accumulation pattern that was found for Cy3-CAG7-OMePS (Suppl. Fig. S4).

\textbf{Interfering with intracellular trafficking using endocytosis modulators}

A significant fraction of the vesicles that incorporated Cy3-DMD23-OMePS or Cy3-CAG7-OMePS stained for the late endosomal/lysosomal marker Lamp1 (Fig. 2A). This suggested that at least a proportion of the vesicles that contained gymnosis-delivered OMePS AONs followed the endocytic route up to lysosomes, confirming earlier observations [20]. To study this pathway more specifically for myoblasts, we examined whether endocytic modulators like chloroquine and dynasore would affect intracellular distribution of DM1 and DMD AONs. Chloroquine is a lysosomotropic agent that leads to endosomal
Figure 1. Live imaging of gymnosis in proliferating DM500 myoblasts. DM500 EGFP myoblasts were cultured for three days in the presence of Cy3-CAG7-OMePS (200 nM). (A) Confocal images showing intracellular localization of the AON (red). Nuclei are green due to expression of a nuclear EGFP-fusion marker protein. Cy3-CAG7-OMePS quickly accumulated in cytoplasmic vesicle-like structures. The AON accumulated in a fraction of the cells in spots in the nucleus (arrowheads). Scale bars indicate 20 µm. (B) Quantification of red fluorescence per cell (n=3; 30 cells per experiment) using images under saturation levels. (C) DM500 myoblasts were grown in the presence of a mixture of Cy3- and FAM-conjugated CAG7-OMePS (200 nM each). An identical vesicular, cytoplasmic staining was observed for both AONs after two days. After four days a clear diffuse staining in cytosol and nucleus was detected in a minority (<5%) of the cell population (arrowheads). Scale bars indicate 20 µm. For visualization purposes, images displayed in (A) and (C) were adjusted to the maximum exposure level within the range of negative background signal.
disruption in vitro [34] and can release AONs from endocytic vesicles [35]. Dynasore inhibits dynamin, a protein essential for clathrin-dependent endocytosis [36].

Dynasore was added one hour before starting the incubation with AONs and kept in culture during the rest of the gymnosis period in order to block clathrin-dependent endocytosis during the entire process. Dynasore treatment completely blocked AON uptake and distribution, supporting the hypothesis that 2′-OMe PS AONs are internalized by clathrin-mediated endocytosis (Fig. 2B; reviewed in [20]). In contrast, chloroquine treatment resulted in strong increase in nuclear accumulation in many cells. Chloroquine was present during the final four hours of gymnosis, to allow AONs to reach all endosomal compartments first. The combination of these observations suggests that upon gymnotic uptake of AONs, endosomal escape is required for subsequent transfer to the cell nucleus.

**Gymnosis in differentiated myotubes**

Gymnosis in non-proliferating cells is reported to be challenging [23]. We therefore studied independently unassisted AON uptake during differentiation of myoblasts to myotubes in vitro. We added Cy3-CAG7-OMePS and Cy3-DMD23-OMePS to differentiating myoblast cultures for up to seven days. As in myoblasts, AONs accumulated in myotubes in cytoplasmic vesicles and multiple myonuclei were AON-positive (Fig. 3). We questioned whether this resulted from intrinsic uptake mechanisms in these differentiated cells or whether they were donated by AON-loaded myoblasts during fusion. To evaluate this point, we performed gymnosis experiments with cell cultures that had first undergone differentiation for three days and were then treated with cytosine β-D-arabinofuranoside (Ara-C) for two days (Fig. 4A). Ara-C, a nucleoside analog that interferes with DNA replication, causes apoptosis of proliferating cells [37] and has been used to remove myoblasts during differentiation to achieve a pure myotube culture [38]. Non-fused myoblasts were largely removed from the adhered myotube layer after Ara-C treatment and this correlated with a significant change in gene expression of several markers reported to be up- or downregulated during myogenesis [39,40] (Suppl. Fig S5). Gymnosis initiated after Ara-C treatment resulted in intracellular AON distribution patterns in myotubes that were indistinguishable from those observed before or in myoblasts (Fig. 3; data not shown). These experiments thus indicate that differentiated myotubes, like proliferating myoblasts, are able to internalize 2′-OMe PS AONs by endocytosis.

**AONs gymnottically delivered during proliferation and differentiation are active in the nucleus**

To analyze whether gymnottically delivered AONs in myoblasts were released from endosomes to become active towards nuclear targets, we first examined the ability of CAG7-OMePS to silence DMPK (CUG)500 transcripts. Silencing of expanded DMPK mRNA in myoblasts could be measured and was further increased in presence of chloroquine, whereas breakdown was prohibited in presence of dynasore (Fig. 2C). In line with earlier observations [7,15], we did not see an effect of the Cy3 modification on nuclear activity, be it conjugated to the 5′ or 3′ end (Suppl. Fig. S3B). Not surprisingly, the bioactive effect of Cy3-CAG7-OMePS on
Figure 2. Intracellular trafficking of AONs during gymnosis. (A) Confocal images of DM500 EGFP cells after 24 hours of growth in the presence of Cy3-CAG7-OMePS or Cy3-DMD23-OMePS. Lamp-1, a lysosomal marker protein, was visualized by immunochemistry and co-localized with some of the AON-positive vesicles. Scale bars indicate 25 µm. (B) Uptake of Cy3-DMD23-OMePS and Cy3-CAG7-OMePS (500 nM) in presence of chloroquine or dynasore after 24 hours of gymnosis. Merged images are a composite of Cy3 and EGFP channels. Scale bars indicate 25 µm. (C) Quantification of expanded DMPK RNA expression after 24 hours of gymnosis incubation with Cy3-CAG7-OMePS in combination with chloroquine or dynasore. DMPK mRNA levels after Cy3-CAG7-OMePS and control AON treatments were compared by unpaired t-tests. CAG7-OMePS activity in presence of chloroquine compared to vehicle was analyzed by 1-way ANOVA followed by a Bonferroni’s Multiple Comparison Test (*: p<0.05, **: p<0.01).
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Figure 3. Gymnosis during myogenic differentiation. DM500 myoblasts were grown to confluency and transferred to differentiation medium in the presence of Cy3-CAG7-OMePS or Cy3-DMD23-OMePS (500 nM). These culture conditions were continued for seven days. After this period, a vesicular cytoplasmic localization was observed in fully differentiated myotubes (live imaging). Clear nuclear signal (arrowhead) could be observed in some myotubes. Compare this faint diffusive staining with total absence of staining in nuclei in other myotubes. For visualization purposes, images displayed were adjusted to the maximum exposure level within the range of negative background signal. Scale bars indicate 20 µm.

DMPK mRNA levels in differentiating myotubes was also sensitive to chloroquine treatment and very comparable between cultures that received Ara-C treatment and those that did not (Fig. 4B).

We next examined the ability of gymnotically delivered DMD23-OMePS to skip exon 23 during DMD pre-mRNA splicing. Since DMD expression, as opposed to that of DMPK, is rather low in myoblasts but strongly induced during myogenic differentiation (data not shown; [41]), we only investigated DMD23 effects in differentiated myotubes. We found that the skipping effect of this AON was the same in Ara-C-treated and untreated myotube cultures (Fig. 4C). Chloroquine treatment enhanced the activity of DMD23-OMePS to levels comparable to those achieved by PEI-mediated transfection. This suggests that intracellular trafficking of AONs in differentiated myotubes is a process that is amenable for modulation, and thus for improvement of its translational value. Finally, we examined exon 23 skipping levels in myotubes after dedicated gymnosis protocols during either proliferation or differentiation or during both periods (see Fig. 5A). DMD23-OMePS-mediated exon skipping was AON concentration-dependent and most effective when applied during the entire period of proliferation and differentiation (Fig. 5B).
Figure 4. Gymnosis in differentiated DM500 myotubes. (A) Experimental protocol used for treatment of myotubes with CAG7-OMePS and DMD23-OMePS. Differentiation of DM500 myoblasts was initiated at 100% confluency at day 0. In protocol T, as a positive control, at day 8 of differentiation myotubes were transfected with AON (500 nM) using polyethylenimine (PEI). Alternatively, cells were allowed to fuse for three days and were then incubated with Ara-C for two days or left untreated (protocols G and Q). At day 5, gymnosis was started and myotubes were incubated with AON (500 nM) for 5 days (protocol G) or for only 3 days after which they were treated with chloroquine (CHQ) followed by the addition of fresh differentiation medium without AON (protocol Q). In all protocols, RNA was isolated at day 10.

(B) Analysis of DMPK mRNA expression (n=3). All groups treated with CAG7-OMePS showed significantly lower levels compared to the matched controls that received DMD23-OMePS (unpaired t-tests; only shown for the transfection pair; **: p<0.01). We also tested whether the effect of CHQ was significant in...
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- each experimental group by t-test analysis (*: p<0.05, **: p<0.01). (C) Analysis of *Dmd* exon 23 skipping (n=3). The gel image shows results of one of the triplicates, while the graph depicts quantification of skipping percentage (n=3). A t-test analysis against a zero theoretical mean was performed in each case to determine whether the average signal of *Dmd* skipped products was significantly different from background (****: p<0.001). DMD23 = DMD23-OMePS; CAG7 = CAG7-OMePS; RT(-) = no RT control; NTC = no template control.

**Effects of AON chemistry on gymnosis delivery and nuclear activity**

In the course of pilot studies, wherein gymnosis was tested for a range of chemically modified CAG AONs, we observed different trafficking results. For example Cy3-CAG3-ENA gave almost undetectable endosomal accumulation [7] (Suppl. Fig. S6). This finding was consistent with previous observations in other cells [24], where AON uptake behavior was strongly influenced by chemical modification. As this aspect is relevant for future therapeutic applications, we tested effects of a selected set of oligo chemistries on gymnosis in our DM1 myogenic cell model. Four modifications for both AON sequences, CAG7 and DMD23, were analyzed: 2’-O-methyl phosphorothioate (OMePS), 2’-O-methyl phosphate (OMe), DNA phosphorothioate (PS) and OMePS including a 5’ conjugation with a muscle-homing peptide (P4), which has been shown to enhance activity of DMD23-OMePS and CAG7-OMePS *in vivo* ([27]; Mulders et al., unpublished). Note that AON DMD23-PS was included to complete the set, but is in fact not useful for therapeutic purposes, since this AON may induce RNAsase-H-dependent breakdown of *Dmd* pre-mRNAs instead of exon skipping.

By using immunofluorescent image acquisition, we were able to quantify Cy3-signals separately for nucleus and cytoplasm in myoblasts (Fig. 6A; note that this protocol could not be applied to myotube cultures). Activity of DM1 and DMD AONs was measured in parallel in seven-day-old myotubes (Fig. 6B). Forty eight hours of gymnosis led to a predominant cytoplasmic location for all AONs, irrespective of the chemical modification and sequence (Fig. 6C). However, a significantly lower uptake was observed for AONs with a phosphate instead of a phosphorothioate backbone, which corresponded to a lack of activity towards their nuclear target (Fig. 6D). Conjugation of peptide P4 to OMePS AONs had an inconclusive effect on gymnosis as it increased uptake of CAG7-OMePS, but had essentially no effect on uptake and cytosolic accumulation of DMD23-OMePS. Assisted delivery of the AONs by PEI transfection led mainly to nuclear localization (Fig. 6E), but with lower efficiency for AONs with only OMe or PS backbones. The tendency of nuclear accumulation is obviously promoted by the well-known facilitating effect of PEI on endosomal escape [42,43].

Nuclear activity clearly correlated with gymnastic uptake efficiency as assessed by cytoplasmic accumulation. The CAG7-OMePS AONs had most, CAG7-OMe least, and CAG7-PS intermediate silencing potential (Fig. 6D). Interestingly, PEI-assisted transfection yielded similar efficiency differences between the OMePS, OMe and PS AONs and the effects on breakdown and exon skipping (with the exception of RNase H recruiting AON DMD23-PS) were moderately improved by PEI transfection (Fig. 6F). Our findings therefore challenge the suggested idea that a strong AON accumulation in the nucleus is required for a robust effect on nuclear targets [44,45].
Figure 5. AON concentration- and time-dependent effects of gymnosis on *Dmd* exon 23 skipping. (A) Protocol used for DMD23-OMePS gymnosis in DM500 myoblasts. Gymnosis was performed during the last two days of proliferation (P), during seven days of differentiation (D) or during both periods (P+D). PEI transfection was carried out as a control on day 5 of differentiation. In all cases, RNA was isolated at day 7 of differentiation. (B) Analysis of *Dmd* exon 23 skipping. A t-test analysis against a zero theoretical mean was performed in each case to determine whether the average signals of *Dmd* skipped products were significantly different from background (*: p<0.05, **: p<0.01). Gel image compares results from gymnosis at 5 µM and transfection with 500 nM DMD23-OMePS. DMD23 = DMD23-OMePS; PEI = polyethylenimine; L = DNA size ladder; NTC = no template control; RT(-) = no RT control.
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Figure 6. Chemistry effects on AON uptake and activity during gymnosis. (A) Masking procedure used to quantify Cy3-fluorescence in nucleus and cytoplasm of myoblasts. Scale bars indicate 20 µm. (B) Protocols used for cell culture and gymnosis and subsequent quantification of AON uptake and
A number of studies have been reported in the past few years on effective treatment strategies using naked AONs in patients and mouse models for DMD and DM1 [6,46,47]. Since the AONs used were rather large, hydrophilic molecules that could not easily diffuse through muscle cell membranes [48], alternative cell internalization mechanisms must have been responsible for the observed biological effect in muscle. One distinct feature with regard to cellular uptake of AONs in vivo specific for DMD is that due to the dystrophin-deficiency some muscle fibers are more permeable to large compounds due to membrane alterations [49], while other fibers retain good membrane integrity [50], thereby directing treatment to the most affected ones [51]. In contrast, in muscles from DM1 patients and mouse models all myofiber membranes appear impermeable to large molecules [52]. Supportive insight concerning normal AON delivery and intracellular trafficking mechanisms may be provided by cell culture studies, since it was shown that cells are able to internalize AONs freely from the culture medium, a process now known as gymnosis [19,23].

We confirm here that gymnosis is a general characteristic of the DM500 myogenic cell culture model. After several hours of incubation in the presence of AONs, vesicular localization patterns were observed in proliferating myoblasts and in differentiated myotubes. Our findings thus demonstrate that myotubes retain AON uptake potential after differentiation and strengthen the hypothesis that similar uptake mechanisms may be at play in muscle in vivo (knowing that differentiated myotubes in vitro exhibit several characteristics of mature muscle myofibers in vivo, like excitation contraction coupling and expression of muscle differentiation biomarkers [53,54]).

The first step in unassisted AON uptake must occur at the cell membrane and involves AON binding and initial engulfment by endocytosis [55]. We found that gymnosis was blocked by dynamin inhibition, which suggests that the endocytic mechanism by which AONs enter cells is clathrin-mediated [20]. Although it is not known how naked AONs are initially recognized and endocytosed in myogenic cells, a phosphorothioate (PS) modified backbone is apparently important, as AONs in our study carrying a phosphate backbone (and either 2'-OMe or ENA ribose modification) were inactive. This idea is supported by a study showing enhanced cellular binding of AONs with PS modification compared to an unmodified phosphate backbone [56]. The importance of PS backbone is further consistent with earlier
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reports on successful gymnosis of a variety of AON chemistries, all carrying this modification [19,24,57,58]. The endocytic process might be triggered by the surrounding proteins that bind to PS AONs in a non-specific manner [59], rather than by the oligonucleotide itself.

In the presence of polycationic transfection reagents, like PEI, the internalization process is mediated by the recognition of positively charged PEI-AON complexes by proteoglycan membrane receptors [60]. Initial steps in unassisted AON endocytosis may be triggered by polyanion-interacting proteins that specifically bind phosphorothioate moieties in AONs [59]. Some of the candidates involved may have been identified in a recent screen for proteins that bind phosphorothioate linkages with high affinity [61]. Normal uptake efficiency of phosphorothioate AONs may be enhanced in vivo by conjugating peptide ligands, as was observed for P4-CAG7-OMePS [17,27,62].

A second important step required to achieve AON activity inside the cell is escape from the endosomal compartment and subsequent trafficking to the target location, i.e. in our study the cell nucleus, where expanded DMPK RNAs and Dmd pre-mRNAs reside. It has been postulated that AONs may follow two distinct endocytic routes [57]: a non-productive route to lysosomal accumulation and degradation, and a productive route resulting in nuclear trafficking and effective RNA regulation. Some of the CAG7-OMePS or DMD23-OMePS AON-positive vesicles indeed co-localized with the lysosomal marker Lamp1, suggesting that the non-productive pathway was followed by a fraction of the internalized AONs. The non-co-localizing CAG7-OMePS/DMD23-OMePS AON fraction may be associated with the postulated productive route and may be present in early endosomes from which they escaped.

The exact mechanism by which AONs escape from endosomes is not known, but it may occur in the course of intracellular trafficking by membrane bilayer instability during vesicle fusion and budding [22]. We found that endocytic release, promoted by chloroquine treatment, indeed enhanced the activity of CAG7-OMePS and DMD23-OMePS towards their corresponding targets. Chloroquine treatment correlated with a switch from a vesicular staining pattern to a predominant nuclear signal, suggesting that soon after endosomal escape free AONs will quickly diffuse through nuclear pores into the nucleus [63,64]. A diffuse nuclear staining including spots has been reported previously after microinjection of AONs carrying a phosphorothioate backbone in the cytosol [63-65].

Despite the weak nuclear and strong cytoplasmic (vesicular) AON staining after gymnosis, a significant activity by both AON sequences was detected towards RNA targets that are clearly located in the nucleus [66,67]. In control experiments, we found that AON activity was higher when PEI-mediated transfection was applied, but the magnitude did not correlate with the higher nuclear AON concentration (estimated at 5-25 times higher, judging from fluorescence quantification; as a result of endosomal release induced by a proton-sponge effect of PEI [42,43]). So, how then can the apparent discrepancy between nuclear AON concentration (fluorescence intensity) and nuclear activity (RNA degradation or exon skipping) after gymnosis be explained?

We propose a few possible explanations, which are not mutually exclusive: (i) PEI molecules may remain associated and form stable complexes with AONs in the nucleus
[68], thus decreasing free AON concentration and impeding proper binding to the mRNA target. (ii) AON concentration in the nucleus during gymnosis may not surpass the limit of detection of our microscope in the majority of cells, but the minimal AON concentration required for a biological effect in this compartment may be much lower than generally believed and is already achieved under these conditions. Nevertheless, it is important to point out that several other factors may influence bioactive efficacy, like levels of target RNA, AON efficiency, speed of the process and whether one AON can modulate multiple transcripts. For instance, CAG7-OMePS AON activities after gymnosis and transfection were more similar than those of DMD23-OMePS, suggesting that the DM1 AON requires a lower concentration in the nucleus. (iii) Related to the previous point, cell imaging has shown that gymnosis is an intrinsic cellular process active in all cells, whereas PEI-mediated transfection involves only part of the cell population (depending on transfection efficiency: around 50% in our experiments). Therefore, the proposed minimal AON concentration in the nucleus for biological effect may be present in a larger portion of the cell population during gymnosis compared to transfection.

In sum, we demonstrate that gymnosis is feasible in proliferating and non-proliferating muscle cells and confirm the relevance of AON chemistry for uptake and intracellular trafficking with this method. Our data suggest that even low levels of AONs in the nucleus may be already sufficient for bioactivity and thus that gymnosis may be a useful method for comparative analysis of therapeutic AON candidates in muscle cells in vitro.

| ACKNOWLEDGEMENTS |
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| AUTHOR DISCLOSURE STATEMENT |
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Free uptake of oligos in muscle cells in vitro

REFERENCES


Free uptake of oligos in muscle cells in vitro


### SUPPLEMENTARY MATERIALS

**Table S1.** Primers and TaqMan® assays used in this study.

<table>
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<th>Name</th>
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<td>mDmd e22-e24 Fw</td>
<td>ATCCAGCAGTCAGAAAGCAAA</td>
</tr>
<tr>
<td>mDmd e22-e24 Rv</td>
<td>CAGCCATCCATTCTGTAGGG</td>
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<tr>
<td>hDMPK e1-e2 Fw</td>
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<td>Mm01326466_m1*</td>
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*Assay ID according to Applied Biosystems (Thermo Fisher Scientific) denomination.

Figure S1. Intracellular AON distribution changes in time after regular formaldehyde fixation. DM500 myotubes grown in the presence of 500 nM Cy3-DMD23-OMePS for seven days were imaged live (compare Fig. 3) and at different time points following formaldehyde fixation, as indicated. Following fixation, AON signal progressively diffused into nuclei (e.g., arrowhead) when cells were kept in PBS. Scale bars indicate 20 µm.
Free uptake of oligos in muscle cells in vitro

Figure S2. Representative AON localization patterns after PEI-mediated transfection. DM500 myoblasts were transfected using PEI with a mixture of Cy3- and FAM-conjugated CAG7-OMePS (each 200 nM) and imaged after two days. The image shows two cells with different uptake patterns. The upper one displays strong nuclear signal (traditionally considered positive for transfection), whereas the lower one presents only faint vesicular staining and essentially lacks nuclear signal (traditionally considered negative for transfection). Scale bars indicate 10 µm.
Figure 53. No effect of the fluorophore position on gymnosis. (A) Localization of CAG7-OMePS, Cy3 conjugated at either its 5’ or 3’ end, after 48 hours of gymnosis or PEI transfection (500 nM) in DM500 EGFP-Mbnl1 myoblasts. Scale bars indicate 25 µm. (B) Quantification of expression of expanded DMPK transcripts after 48 hours of gymnosis with CAG7-OMePS Cy3-conjugated at either its 5’ or 3’ end (500 nM). Data was analyzed by 1-way ANOVA followed by a Bonferroni’s Multiple Comparison Test (***: p<0.001).
Free uptake of oligos in muscle cells \textit{in vitro}

Figure S4. Examples of cellular distribution patterns after gymnosis of Cy3-DMD23-OMePS. DM500 myoblasts were imaged after 24 hours gymnosis incubation with Cy3-DMD23-OMePS (500 nM). A spotted distribution in the nucleus was observed in some cells (top row) and, less frequently, AON signal appeared diffuse throughout the nucleus (bottom row). Scale bars indicate 20 µm.
Figure S5. Validation of Ara-C treatment. (A) Scheme illustrating Ara-C treatment on day 3 to 5 of myogenic differentiation. (B) Representative images of cell cultures acquired at the end of the Ara-C incubation period and four days later. Scale bars indicate 50 µm. (C) Comparison of myogenic marker expression at day 10 of myogenesis (with/without Ara-C treatment). RNA from proliferating myoblasts was taken along as a control (n=6). Myh1, Mylfp and Cav3 expression was induced during myogenesis and was significantly enriched after Ara-C treatment. Aqp1 was mainly expressed in myoblasts, silenced during myogenesis and increasingly lost after Ara-C treatment. For each gene, mRNA levels of untreated and Ara-C-treated myotubes were compared by unpaired t-tests (***: p<0.001).
Free uptake of oligos in muscle cells *in vitro*

Figure S6. Gymnosis of Cy3-CAG7-OMePS versus Cy3-CAG3-ENA. DM500 EGFP-Mbnl1 myoblasts were cultured for three days in the presence of Cy3-conjugated AONs (200 nM). The behaviour of Cy3-CAG7-OMePS (compare Fig. 1) was compared with that of Cy3-CAG3-ENA, a much shorter repeat AON carrying an ethylene-bridged nucleic acid phosphate backbone. (A) Confocal images showing intracellular localization of AONs (red). Cy3-CAG7-OMePS quickly accumulated in cytoplasmic vesicles. In contrast, Cy3-CAG3-ENA was poorly taken up. Scale bars indicate 20 µm. (B) Quantification of red fluorescence (n=3; 30 cells per experiment). Data was analyzed by 2-way ANOVA (**: p<0.01; ***: p<0.001).
Figure S7. Representative images used for quantification of Cy3 signal in nucleus and cytoplasm (see Fig. 6). Images were acquired below saturation limits in the same microscopic session after treatment with the indicated AONs (A) during 48 hours of gymnosis or (B) PEI-mediated transfection. Merged images are a composite of Cy3 and EGFP channels. Scale bars indicate 50 µm.
CHAPTER 7

GENERAL DISCUSSION AND FUTURE PROSPECTS
BACKGROUND

Myotonic Dystrophy type 1 (DM1) is an archetypal member of the group of trinucleotide repeat disorders (TREDs), caused by an expansion of a repetitive DNA sequence of three nucleotides (Chapter 1 – Myotonic dystrophy). DM1 was the first disease ever described for which the underlying cause of the pathology involves an RNA gain-of-function (RNA toxicity) of transcripts coming from a mutated gene (DMPK) [1]. Evidence for RNA being at the center of disease etiology came, among others, from the finding that the mutation itself, an expanded (CTG)n repeat, is expressed as part of the 3’ untranslated region (3’-UTR) in DMPK mRNA transcripts. When swapped by transgenesis to another transcription unit, the repeat can exert its dominant toxic effects as part of other RNA products [2]. Production of expanded DMPK mRNA (or transgenic RNAs) with an expanded (CUG)n repeat leads to nuclear accumulation of the transcript and interferes in trans with normal splicing, polyadenylation and other aspects of RNA processing in all cells of the organism in which the repeat-containing RNA is expressed [3]. Based on this knowledge, there is clear consensus in the DM1 field that the most promising therapeutic interventions should focus on neutralizing the RNA toxicity seen in DM1 patients [4]. Many different strategies are being developed, each trying to reduce the load of expanded DMPK mRNA. From the biological toolbox available for modulation of gene expression, several approaches have been tested, including the use of RNAi [5,6], ribozymes [7-9] and antisense oligonucleotides (AONs) [10-14]. The research described in this Ph.D. thesis is devoted to study AONs of triplet-repeat sequence, complementary to the (CUG)n stretch, as a therapeutic platform for DM1.

TRIPLET-REPEAT AONs FOR THE REDUCTION OF EXPANDED DMPK TRANSCRIPTS

In antisense technology, chemical modifications in the ribose and/or phosphate backbone of the nucleotide sequence are necessary to confer resistance against nucleases, improve pharmacokinetics and enhance binding activity to the target [15] (Chapter 1 – Antisense oligonucleotides). We performed extensive pre-clinical studies in vitro on AONs of different length, sequence and chemical composition in myoblast-myotube cell models derived from patients and from a DM1 mouse model with a human DMPK (CTG)500 transgene (Chapter 2). First, we confirmed the positive aspects of the phosphorothioate (PS) modification to confer stability, since AONs without this type of chemistry showed lower activity. For instance, an AON with 2’-O-methyl (2’-OMe) modified riboses, a chemistry aimed to enhance binding affinity to the target, was much less effective without the PS backbone. In our hands, combining PS linkages with 2’-OMe riboses (2’-OMe PS) resulted in the most effective strategy, as compared to AONs with other chemical modifications like morpholino phosphorodiamidate (PMO) or 2’-O,4’-C-ethylene-bridged nucleic acid (ENA) and to RNAi-mediated knockdown by triplet-repeat siRNAs. We also found that a minimal AON length of five CAG triplets is required to achieve efficient silencing when using 2’-OMe PS chemistry. Furthermore, we performed several experiments with an oligo of (CIG)7 sequence (with inosines instead of adenines). We were interested in this AON because it can hybridize to
(CUG)n, (CAG)n, (CCG)n repeats or any combination of these triplets, making it an attractive candidate for its potential ability to treat multiple TREDs, e.g. DM1, Huntington’s disease and several spinocerebellar ataxias. However, and perhaps not completely unexpectedly, this AON also affected the expression of the endogenous Dmpk transcript, containing a CCG(CUG)2(CAG)2CUG sequence.

As a preclinical step to evaluate AON safety profile, we performed analysis of complement activation in vitro for some of them. We have to consider though, that the predictive value of this test is limited and possible immune responses have to be verified always in vivo. Since methylation of cytosines has been reported to improve the in vivo safety profile of AONs with PS chemistry [16], we used this modification in some of our experiments as well, and found that AONs with the 5-methyl C modification retained equal activity (Chapter 4 and data not shown). All AONs tested in the complement activation assay did not show significant immune stimulation properties except for (CIG)7.

We gained ample evidence during the course of our in vitro experiments that the 2'-OMe PS (CAG)7 AON, binds to the (CUG)n repeat of DMPK transcripts and induce a potent and selective silencing of products from the mutant allele (with or without 5-methylcytosines in the oligo). Furthermore, activity of this type of AON in vivo has been observed in multiple studies with different DM1 mouse models, both after intramuscular injection [11] and systemic administration (Mulders et al., unpublished data). The downstream therapeutic potential has been confirmed by observing that a modest reduction of expanded DMPK transcripts is already sufficient to partially reverse a characteristic gene expression pattern observed in DM1 cells towards normal levels (Chapter 4). Although we do not know the mechanism behind the observed silencing, the activity of these AONs correlates with the length of the target repeat, being more active against long repeats of >100 triplets (Chapter 2) and virtually inactive against normal repeats in DMPK mRNA (usually less than 15-25 triplets) or (CUG)n repeats in transcripts from other genes (typically less than ~12) (Chapter 4). Since repeat length in DM1 also correlates with expected toxicity, we consider this an elegant approach, because careful design of AON chemistry and length can be applied to avoid affecting the expression of normal DMPK mRNA and proteins.

In contrast, we showed that (CAG)n AONs with RNase H activity affect expression of products from both normal and expanded alleles (Chapter 2), reducing also DMPK protein levels. This strategy can thus lead to complete DMPK knock-down, where symptoms associated with DMPK haploinsufficiency could be aggravated. Moreover, RNase H-recruiting AONs also affected expression of other transcripts with shorter (CUG)n repeats, something that could lead to detrimental adverse effects, which are difficult to predict. Interestingly though, clinical trials to evaluate safety, tolerability and efficacy of IONIS-DMPKRx, a gapmer AON with 2’-O-methoxyethyl and constrained-ethyl wings, are currently ongoing. This compound binds to DMPK transcripts outside the repeat (in theory with equal selectivity to both allele products) and makes use of the RNase H mechanism to degrade them. Since this strategy may further reduce the total amount of DMPK protein in cells of DM1 patients, some concerns have been raised regarding the putative detrimental effects of DMPK loss. DMPK insufficiency has been attributed as a possible cause of cardiac conduction defects.
Discussion

in a mouse model for DM1 [17], however, these effects could not be replicated in a recent study that analyzed the conduction system of Dmpk-/- mice or used AON-mediated DMPK knockdown in a DM1 mouse model [18]. Furthermore, cardiac complications could equally well arise from splicing abnormalities caused by MBNL1 sequestration in expanded (CUG)n repeats [19], so be mechanistically non-related to DMPK haploinsufficiency. Nevertheless, relevant effects of DMPK protein loss in other pathobiological manifestations in DM1 should not be dismissed as this protein has been associated with important roles, like Ca\(^{2+}\) modulation in the initial events of excitation-contraction coupling [20], actomyosin assembly [21], nuclear envelop integrity [22], prevention of ROS-induced cell death [23] and myoblast differentiation [24]. In this respect, results from the ongoing clinical trials with IONIS-DMPK\(_{Rx}\) can be expected to shed new light on these questions.

| INVESTIGATING AON-MEDIATED DMPK RNA SILENCING MECHANISMS |
The silencing mechanism of 2'-OMe PS (CAG)n AONs towards expanded DMPK transcripts has remained elusive despite extensive efforts during the studies for this Ph.D. thesis. We consider it unlikely that direct effects on transcription of the mutant DMPK transcripts are mechanistically involved. Treatment with a (CAG)\(_{7}\) AON resulted in a significant reduction of expanded DMPK transcripts already within minutes after transfection (data not shown). Since the average half-life of DMPK mRNA is relatively long [25], inhibition of the already very low de novo production of DMPK (pre-)mRNA [26] cannot explain immediate effects of this AON. Silencing effects by the AON on the steady state levels of DMPK transcripts must therefore have another, but as yet unknown, basis. We explored the most common silencing mechanisms encountered in antisense technology: ribozyme activity, RNase H-mediated knock-down and RNA interference, but found that none of them is directly implicated in the mechanism (Chapter 2). We currently think that AON-induced degradation of the triplet repeat RNA must be mediated by endogenous nucleases present in the cell (similarly to RNase H) or by a yet to be identified mRNA decay pathway. This may happen in the nucleus or the cytosol because, although expanded DMPK mRNA is retained in the former compartment [27], hybridization with (CAG)n AONs may help to export the transcripts by disruption of ribonuclear foci, as has been postulated by others [10].

There is ample evidence that AONs can bind proteins in the cell or upon hybridization to the RNA target [28,29]. This binding is typically dependent on their chemical modifications and the nature of the target itself [29,30]. Proteins recruited by (CAG)n AONs upon binding to the (CUG)n repeat could thus give very important clues about the silencing mechanism. In our pull-down experiments using a biotinylated DMPK mRNA fragment (Chapter 3), we demonstrated that (CAG)\(_{7}\) AON was able to recruit proteins related to RNA processes, which could have implications for the biological fate of expanded DMPK transcripts. Importantly, many of these proteins interacted specifically with the AON-RNA duplex formed upon hybridization of the oligo with the (CUG)n repeat. Ribonucleases and proteins from the DDX family of RNA helicases were significantly enriched in the group of candidate proteins. Potentially, these enzymes could play a role in the silencing mechanism observed after
(CAG)7 treatment. We tested *in vitro* the implications of one of the nucleases identified, FEN1, but failed to detect any RNA degradation products when used in combination with the oligo.

However, many other candidates enriched in the AON-RNA duplex remain to be tested for their possible implication in the mechanism. For instance, several factors listed in Chapter 3 are involved in (pre-)mRNA processing, like splicing regulation, and could potentially play a role. Alternatively, we should also consider the possibility that the key protein involved in the mechanism escaped our pull-down assay, either because the right cellular conditions necessary for binding were not recapitulated or perhaps because the interaction of this protein with the AON-RNA duplex is very transient in nature. For instance, some candidates that we would like to propose to explore in future experiments, but were missed in our interactor search, are several ribonucleases involved in maturation of RNA precursors, like ribosomal RNA (RNase III), tRNA (RNase P) or miRNA (Drosha). These enzymes recognize and cleave certain RNA heteroduplex structures [31,32]. In fact, targeted mRNA knock-down with RNase P is a strategy that has been used in the past [33], usually requiring a so-called external guide sequence [34], which in our case could be represented by the AON. Perhaps, the (CUG)n hairpin formed by expanded DMPK transcripts in combination with (CAG)7 hybridization generates a type of structure that can be recognized and processed by this class of enzymes. A mechanism like this (recognizing the structure rather than the sequence) would also explain the selectivity towards products of the expanded allele, since short (CUG)n repeats have lower tendency to form higher magnitude secondary structures [35,36].

The unexpected finding that (CAG)n AONs can induce exon skipping of transcripts that contain a (CUG)n tract in an internal exon (as in MAP3K4) (Chapter 2) points to the possibility that this type of repeats can work as splicing regulators. In fact, using an *in silico* analysis, a (CUG)n sequence is predicted to bind splicing factors SRSF2 and SRSF6 (data not shown), usually present in exonic splicing enhancers during pre-mRNA processing. Interestingly, SRSF6 is also predicted to bind (CAG)n repeats, where a direct role in the regulation of splicing has been demonstrated [37]. We can speculate then, that AON-induced skipping of MAP3K4 exon 17 was mediated by displacement of these factors from the trinucleotide repeat. The resulting exon17- variant of MAP3K4 retains the normal reading frame. However, a similar approach can be used to trigger nonsense-mediated decay (NMD) when the skipped exon generates an out-of-frame transcript [38]. But in the case of DMPK, we could not find any evidence of splicing in the area of the repeat (Chapter 2). Moreover, since the repeat is located in the last exon, even if a cryptic splice site was used, NMD would not be activated because it requires a newly generated stop codon upstream of the last exon junction [39].

As a relevant example, we have a comparable situation with the HTT gene, where there is a (CAG)n repeat in exon 1 that, similarly to DM1, becomes pathogenic (causing Huntington’s disease) when expanded above certain threshold. In this case, (CUG)n AONs have been used to silence the transcript [40], but also in this study the authors could not unravel the mechanism by which silencing occurred. The mechanisms of DMPK and HTT silencing by triplet repeat AONs may thus share common characteristics and it is tempting to speculate that factors like SRSF2 or SRSF6 could have an important function (other than splicing...
regulation) when binding to nucleotide motifs in the first or the last exon of a transcript. If true, displacement of these factors may lead to a yet-to-identify mRNA decay mechanism. This could be an interesting possibility to study in the future. There is clearly much work to be done to elucidate the mechanism of DMPK or HTT silencing by (CXG)n AONs.

| DELIVERY AND CELLULAR UPTAKE OF AONs |

Another aspect that could improve AON-mediated treatment of DM1 is the cellular uptake of these molecules in the relevant tissues of the patient. This is one of the parameters that may influence treatment success in antisense technology for many diseases [41]. Improving uptake may be challenging due to the fact that AONs are hydrophilic molecules of relatively high molecular weight (generally >7 kDa) [42]. They are most frequently administered systemically in vivo, by subcutaneous or intravenous routes [43]. Nevertheless, AONs with PS backbone have improved pharmacokinetics characteristics compared to their PO analogs or uncharged AONs, which are rapidly excreted from the body [44,45]. This advantage is due to PS binding to serum proteins, something that delays the process of glomerular filtration in the kidneys, resulting in improved tissue biodistribution and availability [46].

For animal models of DM1, it has been reported that AON treatment of muscle tissue can be relatively efficient in vivo, which is associated with a favourable pharmacodynamic profile when systemically administered ([14], Mulders et al. unpublished data). These observations triggered our own study of DM1 membrane permeability (Chapter 5), since we speculated that AON activity in vivo could be due to an abnormal leakiness of muscle fibers, as has been demonstrated in other muscular disorders, like Duchenne muscular dystrophy [47], where lack of dystrophin protein causes loss of muscle membrane integrity [48].

In congenital DM1 patients (the most severe form of DM1) DMD’s exon 78 is aberrantly skipped in ~50% of the transcripts as part of the downstream splicing effects of (CUG)n RNA toxicity [49]. Expression of abnormal DMD could thus affect membrane integrity, however, this remained a speculative possibility as the effect on muscle function of aberrantly spliced DMD exon 78 had not been elucidated until recently. Our analyses described in Chapter 5 showed that DMD expression levels were normal in DM1 patients and mouse models, compared to levels in unaffected individuals and controls. DMD localization in the plasma membrane appeared also completely normal, arguing for a fully functional barrier function of the cell membrane, indistinguishable from controls. Our findings were confirmed in a recent study [50], where other aspects of muscle function were also explored. In a mouse model expressing DMD without exon 78 (lacking the WT DMD gene), the authors observed reduced muscle force, fiber type switch, ringed fibers, sarcoplasmic masses and Z-band disorganization. Although some of these features are also present in DM1 patients, it is in our opinion highly debatable whether this is due to DMD skipping, because DM1 patients still have ~50% of normal DMD expression, and there are other factors of mRNA toxicity that may contribute to these alterations as well. In any case, in neither of the studies signs of muscle leakiness were observed. We confirmed this by studying more robust parameters of membrane permeability, which were negative in all cases (Chapter 5). Taking all this...
together, we concluded that DM1 membrane characteristics are undistinguishable from healthy individuals, both in DM1 mouse models and in patients.

Then, if muscle membranes in DM1 patients are impermeable to large molecules, how could AONs overcome this barrier in the studies that showed efficacy in vivo? We think that we can answer this question based on the mechanisms that myotubes use to internalize AONs in vitro (Chapter 6). When AONs are freely present in the culture medium, cells can actively incorporate them by endocytosis (i.e. by gymnosis, without assisted delivery), accumulating these molecules in vesicles that eventually reach the lysosomal compartment. According to our microscopic observations, AON nuclear levels via this pathway are expected to be low, yet we could clearly demonstrate AON activity on nuclear targets, indicating that the required concentration for biological activity may be lower than generally thought. We thus hypothesize that a similar mechanism of AON uptake must take place in vivo. As mentioned above, once in the circulation, AONs with PS chemistry bind proteins that delay renal filtration thus increasing their half-life in serum [46]. Since transport of plasma proteins to the subjacent tissues is mediated by transcytosis across endothelial cells [51], AONs bound to them could be passively transferred to the muscle interstitium by this process. Next, we propose that AONs will be internalized in muscle myofibers by endocytosis [52], following the same mechanism we observed in cultured myotubes (Chapter 6). A small percentage of AONs in the endosomes has been proposed to escape by unknown reasons, which could be related to membrane bilayer instability during vesicle fusion and budding [53]. Since we demonstrated that a low AON concentration in the nucleus is sufficient for effective silencing, the described process could explain the activity of systemically administered AONs in the previously cited in vivo studies.

From our experiments performed with free AON uptake by cells in culture, we can clearly point to endosomal entrapment as an important drawback to further improve efficacy, because exposure of AONs to the RNA target, located in either nucleus or cytosol, will be limited by this phenomenon. Thus, strategies aimed to facilitate endosomal escape could increase dramatically the therapeutic potential of AONs. We showed that chloroquine, a drug with well-known lysosomotropic properties [54], can be used to release AONs trapped in lysosomes. This release results in a substantially improved nuclear localization and AON activity. Future studies about the use of lysosomotropic compounds like chloroquine in combination with AONs in vivo are of especial interest. Alternatively, other possible strategy would be the combination (or conjugation) of AONs with molecules or protein domains known to facilitate endosomal escape, like those used naturally by certain microorganisms and toxins [55-58].

| FINAL CONCLUSIONS |

In summary, the research described in my thesis deals with multiple aspects of antisense technology which are important to develop AONs as therapeutic agents, with special dedication to key issues like cellular uptake, interaction with proteins or downstream effects in the transcriptome. We focused on the use of AONs as a therapeutic platform for DM1 and, in particular, explored how triplet repeat AONs can be used to silence DMPK transcripts with
expanded repeats. We opened the path to unravel the underlying mechanism of this process by searching for interacting proteins with a possible role in known or novel pathways, something that could be important to understand and improve this silencing strategy in the future, not only for DM1, but potentially also for other TREDs. In addition, by observing the whole-transcriptome consequences of reducing toxic *DMPK* transcripts, we expanded our knowledge about DM1 molecular mechanisms and the expression profile involved.

During our research, we have investigated multiple AON chemical modifications, sequences and targets. As such, many of the findings disclosed here shall be relevant for antisense technology as a therapeutic platform in general. This could be particularly important in regard to mechanisms of cellular uptake and intracellular trafficking, since they appear to be driven mostly by nucleotide chemistry rather than by sequence. Hopefully, my thesis shall be of future value for other studies using AONs, particularly those designed to target expanded trinucleotide repeats in human disease, and be a humble contribution to the efforts of the scientific community in finding a cure for patients suffering from these disorders.
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20. Benders AA, Groenen PJ, Oerlemans FT, Veerkamp JH and Wieringa B (1997) Myotonic dystrophy protein kinase is involved in the modulation of the Ca2+...


THESIS APPENDIXES

THESIS SUMMARY IN ENGLISH
NEDERLANDSE SAMENVATTING
RESUMEN EN CASTELLANO
ACKNOWLEDGEMENTS/AGRADECIMIENTOS
CURRICULUM VITAE
LIST OF PUBLICATIONS
RIMLS PORTFOLIO
Thesis summary in English

Myotonic Dystrophy type 1 (abbreviated DM1, for its Latin name *dystrophia myotonica*) is a genetic multisystem disorder caused by an expansion of a repetitive trinucleotide sequence (CTG-CAG)n in the 3' untranslated region of the *DMPK* gene on chromosome 19. DM1 was the first disease ever described for which the underlying cause of the pathology involves an RNA gain-of-function of transcripts coming from a mutated gene. This repeat in the *DMPK* gene is actually carried by all individuals, but normally contains less than 37 triplets. When the repeat is expanded above this threshold, from one generation to the next, the person carrying it will develop DM1, with an age of onset and severity that loosely correlates with (CTG)n length: the longer the repeat, the sooner symptoms occur and the more severe they are.

Some of the adverse effects of the mutation are known to be independent of the *DMPK* gene context, as they also occur when the expanded repeat is artificially inserted in other genes. After transcription, expanded (CUG)n repeats form stable RNA hairpin structures that are retained in the cell nucleus, where they contribute to large inclusions, so called foci, by abnormal binding of ribonuclear proteins, some of which get sequestered and depleted (e.g. MBNL). Stress responses triggered by the expanded RNA lead to activation of some factors (e.g. CELF1) and inactivation of others (e.g. eIF2A), resulting in loss of cellular homeostasis (ribostasis and proteostasis). The most extensively studied factors, MBNL and CELF1, are antagonistic regulators of splicing and their imbalance leads to a pre-mRNA splicing pattern characteristic for embryonic development that is not normally present in adults. This aberrant splicing pattern leads to several downstream alterations in isoform expression that are responsible for a number of DM1 symptoms. For example, myotonia (delayed relaxation of the muscle after contraction, the most prominent symptom of DM1) is caused by the presence of the embryonic isoform of a protein (CLCN1) responsible for regulating the electric excitability of muscle membranes. In addition, other mechanisms likely play a role in DM1 pathogenesis. An extensive explanation of the fundamental aspects of DM1 (including epidemiology, symptoms, genetics and additional pathomechanisms) is given in Chapter 1 – Introduction to myotonic dystrophy.

There is general consent among experts that removing the load of expanded *DMPK* transcripts and/or avoiding its interaction with ribonuclear proteins would be beneficial for patients. Several studies tried to achieve this goal using different strategies, including treatment with ribozymes, siRNAs, small molecules and antisense oligonucleotides (AONs). During my research, I focused on the use of AONs as a potential therapy for DM1, particularly those directed against the expanded (CUG)n repeat itself, the root cause of the disease. AONs are single-stranded, short nucleotide sequences complementary to RNA or DNA targets in the cell. They are usually chemically modified to improve their stability, binding affinity and pharmacokinetics (necessary aspects for their use as therapeutic agents). In most cases, the phosphate groups linking nucleosides are substituted by phosphorothioates (PS) and other modifications are included in the ribose. For a detailed explanation of these chemical modifications and the development of AONs as a therapeutic platform see Chapter 1 – Introduction to antisense oligonucleotides.
Previous work in our lab identified PS58, an AON of (CAG)7 sequence and 2'-O-Methyl (2'-OMe) PS chemistry, which efficiently silenced expression of expanded DMPK transcripts by an unknown mechanism in cells derived from DM1 patients and mouse models. We continued this work by performing pre-clinical in vitro studies with (CAG)n AONs of different length, sequence and chemical composition. This part is covered in Chapter 2. The combination of a PS backbone with 2'-OMe riboses resulted in the most effective strategy, as compared to AONs with other chemical modifications, like morpholino phosphorodiamidate (PMO) or 2'-O,4'-C-ethylene-bridged nucleic acid (ENA). We also found that a minimal AON length of five CAG triplets is required to achieve efficient silencing with 2'-OMe PS AONs. Perhaps the most important finding was that this type of AON preferentially binds expanded repeats, allowing normal expression of non-expanded DMPK mRNA and other transcripts with shorter repeats. In contrast, RNase H-mediated knockdown led to the silencing of transcripts with repeats of normal size as well, lowering the amount of DMPK proteins in the cell.

In Chapter 2 we also describe several experiments aimed at elucidating the mechanism of action of (CAG)n AONs. Several possibilities were explored: transcription blockage, AON ribozyme activity, RNase H-mediated silencing and RNA interference (Chapter 2 and appendix), but found that none of them are directly implicated. We then speculated that expanded DMPK mRNA knockdown could be mediated by a novel pathway. Chapter 3 describes how we explored this possibility using a pull-down assay with a synthetic DMPK mRNA fragment containing 90 CUG triplets. The RNA was biotinylated and incubated with myoblast proteins and (CAG)7 AON, and then used as bait to pull-down and identify proteins that interact specifically with the AON-RNA duplex by means of streptavidin-coated beads. We found several proteins that may be involved in the mechanism responsible for expanded DMPK degradation, like RNA helicases or ribonucleases, but have yet not confirmed their direct involvement.

In order to assess the effect of (CAG)7 AON treatment on the transcriptome in general, and the effect of reducing expanded DMPK transcripts, we performed gene expression profiling of muscle cells from DM1 patients and unaffected controls. These experiments are described in Chapter 4. Cells were treated with (CAG)7 AON before and after terminal differentiation to myotubes and analyzed by RNA-Seq and SAGE. The data confirmed that no overt side effects in the transcriptome are to be expected from (CAG)7 AON transfection, compared to a control AON with scrambled sequence. In turn, (CAG)7 treatment partially reversed the DM1 expression pattern to that observed in unaffected controls. Of note, this reversal was observed without the expected correction of several characteristic DM1 splicing abnormalities, something that could contribute to a different understanding of the events leading to DM1 pathology.

We then turned to one of the most important aspects of any AON-mediated therapy: efficient delivery. Our first question was whether the cell membranes of muscle myofibers in DM1 patients are permeable to large molecules. Dystrophin splicing has been reported to be affected in DM1, and defects in this protein are associated with loss of membrane integrity in other muscular disorders. Chapter 5 describes the evaluation of cell membrane integrity and dystrophin expression and localization in a number of muscle samples from
DM1 mouse models and patients, which did not reveal any abnormalities. Moreover, no increased permeability to large molecules was detected in mice in vivo.

Since a therapy with AONs would benefit from improved delivery strategies, we looked at the way myoblasts and myotubes take up AONs in vitro (Chapter 6). We tested AONs with different chemical modifications and found, as reported in previous studies, that free uptake is mediated by endocytosis and requires a PS backbone. In the past, efficient uptake of AONs was only considered possible in proliferating cells but, interestingly, we discovered that this also occurs in (non-proliferating) myotubes. We observed AON activity against targets in the nucleus, suggesting that at least part of the molecules escape from endosomes/lysosomes. Importantly, we showed that AON escape from these vesicles can be pharmacologically enhanced, leading to a remarkable increase in nuclear localization and activity.

Finally, an overview of our findings is presented and discussed in Chapter 7. In summary, the research performed for this Ph.D. thesis focused on the use of AONs as a therapeutic platform for DM1 and, in particular, explored how triplet-repeat AONs can be used to silence toxic DMPK transcripts with expanded repeats. This study also opened up the path to unravel the mechanism behind expanded DMPK silencing by identifying proteins that interact with the RNA-AON duplex and may be involved in DM1 pathology, something that could be of future value to better understand and improve this therapeutic strategy. Our findings will also help to improve AON-mediated therapies in other disorders, like those caused by similar trinucleotide expansions, or by improving AON delivery in muscular disorders in general.
NEDERLANDSE SAMENVATTING

Myotone dystrofie type 1 (afgekort DM1, vanwege de Latijnse naam *dystrophia myotonica*) is een genetische multisysteem-aandoening die veroorzaakt wordt door een verlenging van een zich herhalende trinucleotidevolgorde (CTG)n in het 3’ onvertaalde gebied van het *DMPK*-gen op chromosoom 19. DM1 was de eerste ziekte waarvan bekend was dat de onderliggende oorzaak van de pathologie een RNA ‘gain-of-function’ betrof van transcripten afkomstig van een gemuteerd gen. De herhaling in het *DMPK*-gen is aanwezig in alle mensen, maar is normaal gesproken korter dan 37 tripletten. Wanneer de herhaling deze drempel overschrijdt, van de ene generatie naar de volgende, zal een persoon DM1 ontwikkelen, met een aanvangsleeftijd en ernst die min of meer correleert met de (CTG)n-lengte: hoe langer de herhaling, hoe eerder de symptomen optreden en hoe ernstiger ze zijn.

Sommige van de negatieve effecten van de mutatie zijn onafhankelijk van de *DMPK*-gencontext, aangezien deze ook optreden wanneer de geëxparteerde herhaling kunstmatig in andere genen geïntroduceerd wordt. Na transcriptie vormen de geëxpandeerde (CUG)n-herhalingen stabiele RNA-haarspeldstructuren die ophopen in de celkern. Daar vormen ze inclusies, zogenaamde foci, door abnormale binding aan ribonucleaire eiwitten (bv. MBNL), waardoor de effectieve concentratie van deze factoren elders in de cel wordt verlaagd. Stressreacties veroorzaakt door het geëxparteerde RNA leiden tot de activatie van een aantal factoren (bv. CELF1) en de inactivering van andere (bv. eIF2A), wat resulteert in het verlies van cellulaire homeostase (zowel ribostase als proteostase).

De meest intensief onderzochte factoren, MBNL en CELF1, zijn antagonistische regulatoren van het splicingproces en hun onbalans leidt tot een pre-mRNA-splicingpatroon kenmerkend voor de embryonale ontwikkeling, dat normaal gesproken niet aanwezig is in volwassenen. Dit afwijkende splicingpatroon leidt tot veranderingen in isovormexpresie van eiwitten die verantwoordelijk zijn voor een aantal DM1-symptomen. Zo wordt myotonie (vertraagde relaxatie van de spier na contractie, tevens het hoofdsymptoom van DM1) veroorzaakt door de aanwezigheid van de embryonale isovorm van een eiwit (CLCN1) dat verantwoordelijk is voor het reguleren van de elektrische prikkelbaarheid van de spiermembranen. Naast veranderingen in splicing spelen andere mechanismen waarschijnlijk ook een rol in de pathogenese van DM1. Een uitgebreide uitleg over de fundamentele aspecten van DM1 (inclusief de epidemiologie, symptomen, genetica en aanvullende pathomechanismen) is terug te vinden in Hoofdstuk 1 - Myotone dystrofie.

Deskundigen zijn het erover eens dat het verwijderen van de geëxpandeerde *DMPK*-transcripten en/of het voorkomen van de interactie met ribonucleaire eiwitten gunstig voor de patiënt zou zijn. Verschillende studies hebben geprobeerd om dit te bewerkstelligen met verschillende strategieën, waaronder behandeling met ribozymen, siRNA, ‘small molecules’ en antisense oligonucleotiden (AONs). Tijdens mijn onderzoek lag mijn focus op het gebruik van AONs als een potentiële therapie voor DM1, met name gericht op de oorzaak van de ziekte: de geëxparteerde (CUG)n-herhaling. AONs zijn korte enkelstrengs nucleotidesequenties die complementair zijn aan de beoogde RNA- of DNA-strengen in de cel. Ze zijn meestal chemisch gemodificeerd om hun stabiliteit, bindingsaffiniteit en farmacokinetiek te verbeteren (noodzakelijke aspecten
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voor het gebruik van AONs als therapeutische middelen). In de meeste gevallen worden de fosfaatgroepen die de nucleosiden aan elkaar koppelen vervangen door fosforothioaten en worden er tevens andere modificaties aan ribosegroepen aangebracht. Voor een gedetailleerde uitleg van deze chemische modificaties en de ontwikkeling van AONs als een therapeutisch platform zie Hoofdstuk 1 - Antisense-oligonueleotiden.

Eerder werk in ons lab heeft aangetoond dat PS58, een AON met een (CAG)7 volgorde en 2’O-methyl (2’-OMe)-fosforothioaatsamenstelling, op efficiënte wijze de expressie van geëxpandeerde DMPK transcripten voorkomt in cellen afkomstig van DM1-patiënten en muismodellen, door een onbekend mechanisme. We hebben dit werk voortgezet door het uitvoeren van preklinische in vitro studies met (CAG)n-AONs van verschillende lengte, volgorde en chemische samenstelling. Dit onderwerp wordt behandeld in Hoofdstuk 2. De combinatie van een fosforothioaatruggegraat met 2’-OMe-riboses resulteerde in de meest effectieve strategie, vergeleken met AONs met andere chemische modificaties, zoals morfolinofosfordiamidaat of 2’-O,4’-C-ethyleen-gebrugd nucleïnezuur (ENA). We vonden ook dat een minimale AON-lengte van vijf CAG tripletten vereist is voor efficiënte transcriptonderdrukking met 2’-OMe-fosforothioaat AONs. Misschien wel de belangrijkste bevinding was dat deze vorm van AON bij voorkeur bindt aan geëxpandeerde herhalingen, waardoor de expressie van niet-geëxpandeerd DMPK mRNA en andere transcripten met kortere herhalingen niet of nauwelijks gehinderd wordt. Een RNase H-gemedieerde aanpak leidde daarentegen wel tot de onderdrukking van transcripten met (CUG)n-herhalingen van normale grootte en verminderde zo de hoeveelheid DMPK eiwit in de cel.

In Hoofdstuk 2 beschrijven we tevens verschillende experimenten met als doel het ophelderen van het werkingsmechanisme van (CAG)n AONs. Verschillende mogelijkheden werden verkend: transcriptieblokkade, AON-ribozyme-activiteit, RNase H-gemedieerde onderdrukking en RNA-interferentie (Hoofdstuk 2 en bijlage), maar geen van alle bleek rechtstreeks betrokken te zijn. Vervolgens hebben we gespeculeerd dat de afbraak van geëxpandeerd DMPK mRNA zou kunnen worden gemedieerd door een tot dan toe onbekende route. Hoofdstuk 3 beschrijft hoe we deze mogelijkheid onderzocht hebben met behulp van een zogenaamd pull-down experiment met een synthetisch DMPK-mRNA-fragment dat 90 CUG tripletten bevat. Het RNA werd gebioryneerd en geïncubeerd met myoblastewitten en (CAG)7-AON en vervolgens gebruikt als aas voor het identificeren van eiwitten die specifiek binden aan de AON-RNA-duplex met behulp van streptavidine gecoat bolletjes. We hebben een aantal eiwitten gevonden die betrokken zouden kunnen zijn bij het mechanisme dat verantwoordelijk is voor de degradatie van geëxpandeerd DMPK, zoals RNA-helicasen of ribonucleasen, maar hebben hun directe betrokkenheid nog niet bevestigd.

Om het effect van (CAG)7-AON-behandeling op het transcriptoom in het algemeen en op de vermindering van geëxpandeerde DMPK transcripten te evalueren, hebben we genexpressieprofilerings uitgevoerd op spiercellen van DM1-patiënten en gezonde controles. Deze experimenten zijn beschreven in Hoofdstuk 4. De cellen werden voor en na terminale differentiatie tot spiervezels in kweek behandeld met (CAG)7-AON en geanalyseerd met
Appendix

RNA-Seq en SAGE. De gegevens bevestigden dat er geen substantiële bijwerkingen van (CAG)7-AON-transfectie op het transcriptoom te verwachten zijn, vergeleken met een controle-AON met een willekeurige sequentie. Bovendien draaide (CAG)7-behandeling het DM1-expressiepatroon gedeeltelijk terug naar dat van gezonde controles. Het is belangrijk te vermelden dat deze normalisatie waargenomen werd zonder de verwachte correctie van een aantal karakteristieke DM1-splicingafwijkingen, wat zou kunnen bijdragen aan het verkrijgen van een ander inzicht in de gebeurtenissen die leiden tot DM1-pathologie.

Vervolgens hebben we ons gericht op een van de belangrijkste aspecten van elke AON-gemedieerde therapie: efficiënte aflevering van de AON in cellen. Onze eerste vraag was of de celmembranen van de spiervezels in spieren van DM1-patiënten permeabel zijn voor grote moleculen, als gevolg van het ziekteproces. Er is eerder gerapporteerd dat dystrofinesplicing is aangedaan in DM1. Defecten in dit eiwit zijn geassocieerd met het verlies van membraanintegriteit in andere spierziekten. Hoofdstuk 5 beschrijft de evaluatie van celmembraanintegriteit en dystrofine-expressie en -lokalisatie in een aantal spiermonsters van DM1 muismodellen en patiënten. Hierbij werden geen afwijkingen geconstateerd. Bovendien werd er geen toegenomen permeabiliteit voor grote moleculen gedetecteerd in muizen in vivo.

Aangezien een behandeling met AONs zou profiteren van betere afleverstrategieën in cellen, hebben we gekeken naar de manier waarop spierstamcellen (myoblasten) en spiervezels AONs opnemen in celkweek (Hoofdstuk 6). We hebben AONs met verschillende chemische modificaties getest en vonden dat, zoals reeds is gemeld in eerdere studies, vrije opname wordt gemedieerd door endocytose en dat hiervoor een fosforothioaatruuggengraat vereist is. In het verleden werd efficiënte opname van AONs alleen in prolifererende cellen mogelijk geacht, maar interessant genoeg ontdekten we dat dit ook gebeurt in (niet-prolifererende) meerkernige spiercellen in celkweek. We zagen AON-activiteit tegen RNA-doelen in de cellkern, hetgeen suggereert dat tenminste een deel van de moleculen ontsnapt uit de endosomen/lysosomen. Belangrijk is dat we aangetoond hebben dat het ontsnappen van AONs uit deze blazenjes farmacologisch kon worden verbeterd, wat leidde tot een opmerkelijke toename in nucleaire lokalisatie en activiteit.

Tot slot wordt er een overzicht van onze bevindingen gepresenteerd en bediscussieerd in Hoofdstuk 7. Kortom, het onderzoek uitgevoerd voor dit proefschrift was gericht op het gebruik van AONs als therapeutisch platform voor DM1. Er is met name onderzocht hoe AONs met een zich herhalende trinucleotidevolgorde kunnen worden gebruikt om toxische DMPK transcripten met geëxpandeerde herhalingen te neutraliseren. Deze studie opende ook de weg naar het ontrafelen van het mechanisme hierachter, door het identificeren van eiwitten die een interactie vertonen met het RNA-AON-duplex en dus mogelijk bij de DM1-pathologie betrokken zijn. Deze resultaten kunnen in de toekomst nuttig zijn bij het begrijpen en verbeteren van deze therapeutische strategie. Onze bevindingen zullen ook helpen bij het verbeteren van AON-gemedieerde therapiën voor andere aandoeningen die worden veroorzaakt door trinucleotide expansies of door het verbeteren van AON-aflevering aan cellen in spieraandoeningen in het algemeen.
Distrofia miotónica tipo 1 (DM1), es una enfermedad genética dominante y multisistémica causada por la expansión de una secuencia repetitiva de tres nucleótidos (CTG)n en el gen DMPK, localizado en el cromosoma 19. Esta mutación del ADN no está presente en la proteína, pues se encuentra en una zona del gen no codificante (3’-UTR por sus siglas en inglés). DM1 fue la primera enfermedad descrita con una mutación que, tras ser transcrita como una repetición (CUG)n al ARN, produce que éste adquiera por sí mismo una nueva función dañina para la célula. La repetición en el gen DMPK se da en todos los individuos, pero normalmente contiene menos de 37 trinucleótidos. Solo cuando se expande por encima de este umbral (lo cual ocurre, usualmente, de una generación a la siguiente) la persona portadora desarrollará DM1, con una edad de aparición y severidad asociadas a la longitud de la repetición: a mayor número de (CTG)n, generalmente los síntomas aparecen a edad más temprana y son más severos.

Algunos de los efectos adversos de la mutación son independientes del locus de DMPK, ya que también se manifiestan cuando la expansión de trinucleótidos es insertada artificialmente en otros genes. La repetición (CUG)n en el ARN forma una estructura secundaria estable que se une a ribonucleoproteínas (ej. MBNL) que se mantienen secuestradas y contribuyen a la formación de agregados que quedan retenidos en el núcleo de la célula. Esto produce una respuesta de estrés celular por la que algunos factores se activan (ej. CELF1) y otros se inactivan (ej. eiF2A), llevando a una situación de pérdida de homeostasis. La alteración molecular más estudiada en el contexto de DM1 es el splicing o empalme del ARN celular. MBNL y CELF1 son factores que regulan de manera antagónica este importante proceso. Su desequilibrio hace que se produzca un patrón de splicing en la célula que es característico de la etapa embrionaria, pero al mantenerse tras el nacimiento provoca varios de los síntomas asociados a DM1. Por ejemplo, la miotonía (el síntoma más característico en DM1, que consiste en el retraso de la relajación muscular tras la contracción) es causada por la presencia de la isoforma embrionaria de una proteína (CLCN1) responsable de regular la excitabilidad eléctrica de las membranas musculares. Además de splicing, es probable que otros mecanismos contribuyan al desarrollo de la enfermedad. Para una explicación más extensa (en inglés) de los aspectos fundamentales de DM1 (incluyendo epidemiología, síntomas, genética y otros mecanismos de la patogénesis) ver el Capítulo 1 (Chapter 1 – Introduction to myotonic dystrophy).

Entre los expertos en DM1 existe el consenso que reducir la cantidad de moléculas de ARN con la expansión y/o evitar su interacción con ribonucleoproteínas serían estrategias terapéuticas prometedoras. Varios laboratorios han intentado conseguir esto con diferentes métodos, incluyendo tratamiento con ribozimas, siRNAs, pequeñas moléculas y oligonucleótidos anti-sentido. Durante mis investigaciones, me he dedicado al uso de estos últimos como un posible tratamiento para DM1, especialmente aquellos dirigidos específicamente contra la repetición (CUG)n en el ARN, causa directa de la enfermedad. Los oligonucleótidos anti-sentido (a los que me referiré simplemente como “oligos” de ahora en adelante) son pequeñas secuencias de nucleótidos complementarias a partes del ARN o del ADN que se quieren modular. Normalmente se modifican químicamente para
mejorar su estabilidad, fuerza de hibridación y farmacocinética (aspectos imprescindibles para su uso como agentes terapéuticos). En la mayoría de los casos, los grupos fosfato que unen los nucleósidos son sustituidos por fosforotioatos (PS por sus siglas en inglés) y otras modificaciones se incluyen en la ribosa. Para una explicación detallada (en inglés) de estas modificaciones químicas y el desarrollo de oligos como plataforma terapéutica en general ver el Capítulo 1 (Chapter 1 – Introduction to antisense oligonucleotides).

En un estudio realizado previamente en nuestro laboratorio se identificó PS58, un oligo de secuencia (CAG)7 y con la modificación química 2'-O-Metil (2'-OMe) PS, capaz de silenciar la expresión de DMPK mediante un mecanismo desconocido. Mi tesis comenzó con la continuación con este trabajo, realizando estudios pre-clínicos en modelos celulares, tratándolos con oligos de diferentes longitudes, secuencias y modificaciones químicas, todos dirigidos contra la repetición (CUG)n. Esta parte está cubierta en el Capítulo 2, donde se demuestra que la combinación de PS con 2'-OMe ribosas es la estrategia más efectiva al compararla con el uso de otras modificaciones químicas como morfolino fosforodiamidato (PMO por sus siglas en inglés) o 2',4'-C-ethylene-bridged nucleic acid (ENA). También descubrimos que para ser efectivo, la longitud mínima de un oligo (CAG)n debe ser de 5 tripletes. Pero probablemente el hallazgo más importante es que este tipo de oligos se unen preferentemente a repeticiones expandidas, permitiendo la expresión normal del alelo no expandido de DMPK y de otros genes que también contienen repeticiones (CTG)n más cortas. Esto ofrece una gran ventaja frente a otro tipo de oligos que actúan con un mecanismo diferente (mediado por la enzima RNasa H), los cuales también afectan la expresión de genes con repeticiones de longitud dentro del rango normal, incluyendo el alelo no expandido de DMPK. Esto lleva a una menor producción de la cantidad de proteínas de DMPK en la célula, que potencialmente podría tener efectos adversos, pues la proteína DMPK se ha relacionado con importantes funciones fisiológicas.

En el Capítulo 2 también se describen algunos experimentos en los que intentamos resolver el mecanismo de acción de nuestros oligos. Varias posibilidades fueron investigadas: bloqueo de la transcripción, actividad como ribozima, silenciamiento mediado por RNasa H y ARN interferente (Capítulo 2 y apéndice del mismo). Tras comprobar que ninguno de estos mecanismos está implicado, especulamos que el silenciamiento del ARNm expandido de DMPK podría estar mediado por una nueva vía desconocida por el momento. En el Capítulo 3 se describe cómo exploramos esta posibilidad con experimentos en tubo de ensayo. Para ello usamos un fragmento del ARNm de DMPK con 90 tripletes CUG, que fue biotinilado e incubado con el oligo PS58 y proteínas de mioblastos. El ARN fue usado entonces como “cebo” para extraer (por medio de bolitas magnéticas funcionalizadas con estreptavidina) proteínas que interactúan específicamente con el dúplex formado entre oligo y ARN. Usando este método, identificamos varias proteínas que podrían mediar en el mecanismo de degradación de DMPK, como helicasas del ARN o ribonucleasas, pero no hemos podido confirmar todavía la implicación de ninguna de ellas.

Después, con el objetivo de analizar el efecto de oligos (CAG)7 en el transcriptoma en general y las consecuencias de reducir ARNm de DMPK expandido, realizamos un estudio de expresión génica en células musculares de pacientes con DM1 y controles no
afectados. Estos experimentos se describen en el Capítulo 4. Las células fueron tratadas con oligo (CAG)7 antes y después de la diferenciación a miotubos y se analizaron con dos modernas técnicas de análisis genético (RNA-Seq y SAGE). Los resultados confirmaron que en el transcriptoma no se observan efectos secundarios de consideración debidos al tratamiento. De hecho, éste oligo es capaz de corregir parcialmente la expresión génica característica de DM1, acercándola a los niveles observados en las células de personas no afectadas. Inesperadamente, esta corrección se produjo sin cambiar las anormalidades de splicing características de la enfermedad, algo que tal vez podría apuntar a diferencias causativas en el modo que entendemos la patología de DM1.

A continuación nos centramos en uno de los aspectos más importantes de cualquier terapia con oligos: su transporte a los tejidos afectados del organismo. Empezamos investigando la membrana de las células musculares en DM1, para averiguar si son permeables a grandes moléculas como los oligos. La razón por la que sospechamos de dicha permeabilidad es debido al splicing incorrecto de una proteína (distrofina) en DM1, que es responsable de mantener la integridad de la membrana muscular. En el Capítulo 5 se describe una evaluación exhaustiva de la expresión y localización de distrofina y la integridad de la membrana celular en DM1, por medio de muestras de modelos de ratón y pacientes. Este análisis reveló que no existen anormalidades en la membrana muscular. Además, experimentos en los modelos de ratón más importantes en DM1 confirmaron que no hay permeabilidad a grandes moléculas in vivo.

Una vez que confirmamos la integridad de las membranas musculares en DM1, y dado que una terapia con oligos se vería beneficiada por una mejora en el transporte al interior de la célula, nos pusimos a investigar cómo estas moléculas son incorporadas in vitro por mioblastos y miotubos (Capítulo 6). Analizamos oligos con diversas modificaciones químicas y observamos que, tal como se ha descrito en estudios anteriores, la internalización es mediada por endocitosis y requiere la modificación PS. Sin embargo, hasta ahora se consideraba que una internalización eficiente de oligos (sin métodos artificiales para atravesar la membrana celular) sólo era posible en células en proliferación, pero descubrimos de manera inesperada que esto también ocurre en miotubos, que son células que no se dividen. Podemos detectar actividad de los oligos en el núcleo de estas células, sugiriendo que al menos parte de las moléculas escapan de endosomas/lisosomas. Además, demostramos que es posible facilitar la salida de los oligos de estas vesículas usando ciertos fármacos, algo que se traduce en un incremento notable de la localización nuclear y actividad.

Para concluir la tesis, una visión de nuestros hallazgos en conjunto se presenta y analiza en el Capítulo 7. En resumen, las investigaciones desarrolladas durante mi doctorado se han centrado en el uso de oligos como una posible plataforma terapéutica para DM1, particularmente estudiando aquellos de secuencia (CAG)n, que pueden usarse para silenciar y reducir la toxicidad del ARNm expandido de DMPK. También hemos abierto el camino para identificar el mecanismo responsable de este silenciamiento al descubrir una amplia colección de proteínas que interactúan con el dúplex formado entre oligo y ARN. El estudio de estas proteínas, que podrían estar involucradas en la patología de DM1, puede ser de gran valor en el futuro para entender mejor y desarrollar más la estrategia terapéutica. Finalmente,
el conjunto de nuestros hallazgos pueden ayudar también a progresar en terapias con oligos para aplicaciones distintas, como otras enfermedades causadas por repeticiones similares de nucleótidos, o para mejorar el transporte de estas moléculas en afecciones musculares en general.
| ACKNOWLEDGEMENTS/AGRADECIMIENTOS |

Right after I started my PhD project, my father gave me a present: Stephen Hawking’s book entitled “On The Shoulders of Giants. The Great Works of Physics and Astronomy”. This book was very inspiring to me. It describes how scientists stand on the knowledge (“shoulders”) of their predecessors and, by doing so, they are able to see further and fill empty gaps with their imagination and experiments. Although this concept exists at least since the 12th century, I doubt many people back then could imagine the astonishing way science works today… we are constantly climbing on the shoulders of fellow contemporary scientists in our field, advancing knowledge at a pace that has no precedent in human history. Far from the intention of comparing myself with one of the greatest scientists in history (that’s not the idea of this paragraph!), I have to acknowledge that this piece of work wouldn’t have been possible without climbing on the shoulders of some “giants” that I encountered during my scientific career so far. In the next paragraphs I will acknowledge them, in rough chronological order.

I know for a fact that I wouldn’t have chosen a scientific path without the inspiration from two teachers during high school. My physics teacher, Elías Fernandez, opened my eyes to the meaning of numbers and formulas behind basic concepts of nature, and how they can be applied to understand the workings of the Universe. My biology teacher, José Luis Hidalgo, inspired me to start a career in biochemistry. I became completely captivated when I realized that a living organism can be generated based on a language (the genetic code), that was created in a way that remains a mystery to us.

After graduating in Biochemistry at the University of Zaragoza, I went for a short stay in the Biochemistry department of King’s College London, where Richard Cammack welcomed me very warmly. I have to thank him very much for his continuous support and kind efforts to make me feel in London and his department like at home. Gracias también a los Xotarros: Elena, Pedro, Alba, Javi, Xabi, Jara… No hubiera sobrevivido Londres sin vosotros y no os voy a olvidar jamás!

My adventure in the Netherlands started thanks to Begoña Aguilera. She is the person who brought me to Prosensa Therapeutics (now BioMarin Nederland) for an internship under her supervision. She trusted me from the first day and made every possible effort to make my stay abroad very comfortable. Begoña, ¡muchísimas gracias, sin ti nunca hubiera tenido la oportunidad de hacer el doctorado y siempre te voy a estar agradecido por ello! After this internship, I asked Judith van Deutekom about the possibility of starting a PhD. She promised me she would do anything possible to make that happen, and kept her word. In addition to that, she has always been encouraging and supporting me. Judith, I still remember the trip when we were returning from my first meeting in Nijmegen about the PhD. You noticed that I was overwhelmed by the challenge and encouraged me by saying that it was going to be hard, but that the experience would be worth it because I was going to learn a lot. Well, indeed, I have learned a lot (including from you) and I have grown as a person thanks to the PhD, so thank you very much for giving me this opportunity. I also need to thank all the people in Prosensa/BioMarin who supported me, especially in those difficult beginnings: Ronald, Tatyana, Galyna, Emilio, Rani, Anneke, Suzanne, Jessica, Niki,
Appendix

Jeroen, Rick, Ruurd, Rudie, Janwillem, Lena, Aabed… and many others of the Discovery department and the company in general, thank you very much! Peter, thank you for your kindness, sense of humor and support. I also appreciate that you came all the way to Nijmegen when we invited you to give a seminar. I am very grateful for your advice and very valuable discussions about oligonucleotide chemistry, and for your corrections and help in preparing my thesis manuscript. Nicole, thank you for your help in the last stage of my PhD and for your understanding and continuous support.

Once I started my PhD in Nijmegen I had to face many challenges: I had to move to a new city again, commute from Utrecht every day and fit in a new lab. To make things worse, my experiments were not working in this critical starting period where I needed to prove that I was up to the challenge… I now realize how lucky I was with the supervisors I had, because they were the most important people who made this accomplishment possible: Rick Wansink (co-promotor), Bé Wieringa (promotor) and Susan Mulders (supervisor from Prosensa). Rick, I couldn’t thank you enough for welcoming me in the lab so warmly, for your patience, support and guidance. I remember when you were joking about being too fatherly with me. Well, you have definitely been my “scientific father” during this time, and you trusted me continuously and encouraged me to gain the self-confidence that I was lacking. I have learned a lot from you, thank you very much for everything! Susan, thank you for letting me climb on your shoulders to continue the work that you started, for helping me so much and teaching me everything. I also appreciate your support, patience, sense of humor and easy-going character, all those characteristics in you as my supervisor helped me overcome many challenges of my PhD. Bé, it has been an honor to be your student, I admire you very much as a person and scientist. I believe you are one of those scientists (one of those “giants”) that are in danger of extinction today: purely driven by curiosity and rigorous scientific method, rather than by the triumphalism of publishing papers and popularity. Moreover, you always keep your feet on the ground, and you are very kind with everyone around you. You inspired me a lot, and your suggestions and advice during our meetings were fundamental to achieve my PhD.

Ingeborg, my friend, thank you very much for everything, you know that your help has been very important for my PhD, I don’t have to tell you that… And thanks for being my paranymph as well! I also acknowledge Walther and Huib (señor Huib!) for teaching me and helping me with so many techniques that I used during my PhD. Thank you to all members of Cell Biology and Animal Physiology who helped me in some way or another with discussions, tips or just for being there: William, Jack, Frank, Jan, Marieke, Irene, Remco van H., Mirthe, Lieke, Anke, Ellen, Laurène, Remco van C., Leontien, Julia O., Cornelia, Gert-Jan, Sjoerd, Jan-Hendrick, Linda, Nick, Eric, Astrid and many others! I am very grateful to the students I guided during my PhD: Ingeborg, Julia K. and Bram, the data you generated were essential to complete my thesis. Thank you Willeke and Melvin for the discussions and help in the projects we collaborated.

During my PhD I have been lucky enough to meet many people and make very good friends. I must acknowledge them as well, because without their support I couldn’t have made it, no doubt about that. There is no order of importance here, I hope my words
will speak by themselves, but I have to start with three friends who were with me from the beginning and are like family to me: Angela, Antoine and Ganesh. Guys, when I left Spain I never imagined that I was going to be part of an Italian, a Lebanese and an Indian family! And look at us now… we consider each other’s parents, brothers and sisters as our own, as part of one and the same family! Think about it, how amazing that is!! One of us once said that when we are far from home we need to share the warmth between the families of our friends (or something like that :P), and this is something that we have done far beyond my expectations. So, please extend my gratitude to all of them, because there is no doubt that I have felt like at home at all times. I could never thank you enough for this, and I would have never made it without you guys!

Toni, there is nothing I could write here that I haven’t told you already in person, bro. You know it. Let me only tell you that, even if I hadn’t succeeded with my PhD, coming to the NL would have been worth it just to become your friend. In addition, you have contributed a lot to my work by helping me with techniques like microscopy and Western blot, and by your great ideas when we were discussing about science in the late evenings. And many thanks for being my paranymph!

Angy, my friend, thank you so much for taking me as part of your family. You have made me feel like at home thanks to your warmth and cheerful energy and that has helped me a lot, especially when we were living together. You were always able to understand what was going through my mind in a way that sometimes was even scary, and you encouraged me and supported me with very good advice!

Gano, we have been through so many experiences in the past years that I will never forget the time we spent together. Thank you for your loving kindness. I’m very glad you invited me to India, not only because it was lovely to celebrate your wedding with Ramya and your united family, but also because now I understand many things about you that I didn’t before. I will see you in another life, brother.

Sip, my favorite Dutchman. You are also like a brother to me. Thank you for all your support. When you are abroad, any help from a local friend is priceless, and I think you know that very well. Thank you for being there, for our crazy adventures, your kindness and your advices. I am also grateful for the Dutch lessons, and for helping me translate the Dutch summary of the thesis (unfortunately I wasn’t such a good student during those lessons to be able to translate it myself hehehe!). Nuria M., gracias a ti también por todo, chiqueta! Te quiero mucho y no sabes cómo os echo de menos!

Cindy D., thank so much for your friendship and support during my PhD, especially at the last moment, when I felt that everything was falling apart. I could only continue standing thanks to the support of my family, Antoine, Bart, André and you. I will never forget that. Thanks for checking the Dutch version of my thesis summary as well.

Fede, Sergio, Elisa, Joanna, Tomasz, Silvieta, Claire… Thank you so much for all the time we spent together, I will never forget you my friends. We started our adventure together in the NL and we made our little expat community with Toni, Angela and Ganesh, playing interminable dart games at Derde Walstraat and partying with DJ Seco. I also learned a lot from you guys, since I was at the beginning of my PhD and most of you were
more experienced. Thank you for the “moustache evening” and many others, Lauriane! Olga, Pavel, Harsha, thank you for your support and for our lovely trip to the “Dutch Venice” :)

Rocio A., Markus, André, Katia, Davide, Vicky, Benny, Niccolò, Stefania, Till, Sandhya, Marco, Pedro, Cindy R., Simone, Philipp… Together with Sip, Nuria M., Ganesh, Antoine and Angela, we formed what for me has been a “second generation” of expat family. It was an amazing adventure with moustache parties, new relationships, Oktoberfest, love, craziness, weddings, babies, feelings, laughter, tears and even some non-fatal blood!

I am very grateful to Blending Voices for bringing me back to theatre and all the members of the group for listening and sharing so many stories. Thank you to Radbal, the Nijmegen basketball group and my coaches Davide and Michael for helping me improve in this sport that, like theatre, helped me so much to disconnect from the stress of the PhD.

Now I will spare a few words in Spanish for my family and friends in Spain

Ahora unas palabras en castellano para mi familia y amigos
Esta tesis va dedicada a mis padres y a mi hermano. Este trabajo también es vuestra, por haberte hecho la persona que soy. Porque nuestros padres nos transmiten mucho más que genes: también heredamos de ellos habilidades, cultura y sensibilidades. En mi caso, la paciencia y curiosidad se la debo a mi madre Malen, y la insaciable perseverancia a mi padre Ángel. A mi hermano también le debo mucho: el hermano mayor no es el único que abre camino, en mi caso Jorge me ha abierto los ojos sobre muchas cosas en la vida. Por todo esto y mucho más os quiero con locura. Este éxito os lo debo a vosotros. Muchas gracias también a Amparo, Luis, Maribel, Paco y Jordana.

Rocio A., gracias por ser mi compañera de viaje durante parte del camino, nunca olvidaré los buenos momentos que tuvimos y lo mucho que me apoyaste para seguir adelante hasta que ya no pudiste más. Agradezco mucho las correcciones y sugerencias que hiciste en varias partes de la tesis.

He tenido que sacrificar muchas cosas en este doctorado, pero sin duda lo más duro ha sido tener que irme lejos de tantos familiares y amigos que quiero. Sin embargo, lo más bonito ha sido darme cuenta de que, a pesar de la distancia, todos esos buenos amigos jamás se han olvidado de mí y cada vez que nos reencontramos parece que no ha pasado el tiempo. Muchas gracias Etien, Nacho, Iván, Darío, Yorch, Kri, Diana, Pedro Pablo, Lauri, Diego, Carlos Podría llenar cientos de páginas con todo lo que cada uno de vosotros me habéis aportado en la vida, pero creo que no hace falta porque todos lo sabéis muy bien. Estoy profundamente agradecido a todos los miembros del Lokal por esos momentazos y fiestones en aquel super garito y mantener nuestra amistad siempre, ¿quién hubiera dicho entonces que llegaría a ser doctor, eh lokers?? Gracias al equipo del Posturas darts y a todos los que pasaron por el equipo (en Holanda todo el mundo sabe de mi pasado dardero). Finalmente, como no podía ser de otra manera, gracias a toda la gente de Pancrudo, el pueblo donde me crié, especialmente a mi inseparable pandilla: Ana, Fernando, Rubén, Nuria V., Jakeko, Gonzalo, Jorge V., Sonia, Clara, Erica, Dani, Carol y vuestros
Acknowledgements/Agradecimientos

respectivos! Os quiero chicos, tampoco hace falta que os diga todo lo que me habéis aportado desde pequeño

Gracias a todos los que confiaron en mi para conseguir este logro, y también a los que no lo hicieron, porque también me motivaron a seguir adelante para demostrar que se equivocaban.

Thank you to everyone who trusted me to achieve this, and also to those who didn’t because they also motivated me to continue.
CURRICULUM VITAE

Anchel González Barriga was born in Zaragoza (Spain) on 20 November 1982. He studied at the University of Zaragoza and obtained a bachelor’s degree in Veterinary Sciences in 2004 and a master’s degree in Biochemistry in 2006. As part of his master’s studies, Anchel worked at the Nanoscience Institute of Aragón (INA for its Spanish acronym) under the supervision of Dr. Ana Isabel G. Lostao, where he implemented surface functionalization techniques to study ferritin and avidin proteins at the nanoscale level by Atomic Force Microscopy. In 2007, Anchel travelled with an Erasmus grant to study for a postgraduate master’s in Molecular and Cellular Biology in the United Kingdom. He joined the lab of Prof. dr. Richard Cammack and Prof. dr. Andrew McKie in the Biochemistry department of King’s College London to study the role of the iron reductase Dcytb cytochrome on the plasma membrane of erythrocytes isolated from human and sheep blood. Anchel received a top grade for his project at King’s and later obtained an Argo mobility grant to perform an internship in a company abroad. He joined Prosensa Therapeutics in January 2009, a Dutch spin-off biotech company based in Leiden that focuses on the treatment of rare genetic disorders by antisense oligonucleotides. After finishing his internship, Anchel was offered the possibility to start a PhD in collaboration with the group of Dr. Rick Wansink and Prof. dr. Bé Wieringa in the Cell Biology department of the Radboud Institute for Molecular Life Sciences (RIMLS) in Nijmegen. During the PhD trajectory, Anchel studied molecular and preclinical aspects of antisense oligonucleotide treatment for myotonic dystrophy type 1. His findings are presented in this thesis. He followed several courses and seminars, including the Radboudumc-CDL animal laboratory course to obtain authorization to perform animal experiments in the Netherlands and a course in qPCR and statistical analysis in Paris. He supervised three undergraduate students and attended multiple international congresses, where talks and poster presentations were given about his work on DM1. Anchel has successfully followed the PhD training program of RIMLS (see portfolio included in this thesis) and he was recently awarded with the RIMLS PhD certificate. As of 2016, Anchel is working as a scientist in BioMarin Nederland (formerly Prosensa Therapeutics), where he is guiding a team trying to find an antisense oligonucleotide-mediated cure for Huntington’s disease, a devastating neurological disorder.
LIST OF PUBLICATIONS


*Equal contribution
Appendix

| RIMLS PORTFOLIO |

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<th>PhD period: 01-01-2010 – 10-08-2016</th>
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<tr>
<td>Department: Cell Biology</td>
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| TOTAL | 35.25 |

^Indicate oral (*) or poster (#) presentation