12-2014

Redesign of trans-splicing molecules for the correction of dystrophia myotonica type 1 toxic RNA transcripts

Eleanor G. Harrison
East Tennessee State University

Redesign of trans-splicing molecules for the correction of dystrophia myotonica type 1 toxic RNA transcripts

Thesis submitted in partial fulfillment of Honors

By

Eleanor G. Harrison

The Honors College

Midway Honors Program

East Tennessee State University

December 7, 2014

Eleanor G. Harrison, Author

Dr. Zachary Walls, Faculty Mentor

Dr. Ismail Kady, Faculty Reader

Dr. Aruna Kilaru, Faculty Reader

ACKNOWLEDGEMENTS

Research was performed under the guidance of Dr. Zachary Walls, Pharmaceutical Sciences, Gatton School of Pharmacy, and funded in part by East Tennessee State University's Student-Faculty Collaborative Research Grant. Editorial support and revision was supplied by Dr. Aruna Kilaru, Department of Biological Sciences, and Dr. Ismail Kady, Department of Chemistry at East Tennessee State University.

Disclaimer: It is to be noted by the reader that, although the procedure for the creation of these PTMs is included within this manuscript for the sake of completeness, these vectors were assembled prior to the assumption of this project by the writer of the report and was not part of the laboratory work conducted by her person.

ABSTRACT

Dystrophia myotonica (DM1), one of the most common forms of muscular dystrophy, is caused by a repeated trinucleotide expansion in the DMPK gene. This mutation results in the accumulation of toxic cellular RNA transcripts. Spliceosome-mediated RNA trans-splicing (SMaRT) technology is a form of gene therapy that possesses the potential to correct these toxic RNA transcripts and thus cure the disease. Despite its promise, prior applications of SMaRT technology to DM1 have been hampered by poor efficiency and have not been validated in a relevant model of the disease. In order to improve the efficiency of trans-splicing, this study examined the use of novel SMaRT molecules containing altered binding domains. These SMaRT molecules were tested in a clinically relevant cell model of DM1 and their corrective ability compared to that of a standard SMaRT molecule. The results were quantified by RT-PCR. The outcome of this study indicated the need to utilize more specific methods for measuring efficiency and for understanding the specific interactions of SMaRT molecules with target transcripts.

INTRODUCTION

1. DM1 overview

The foundation set in the mid-20th century regarding the molecular basis of heredity resulted in a new perspective into disease etiology and pathology. With the completion of the human genome project and the systematic mapping of genes in the early parts of the 21st century, many disease states could, for the first time, be linked to genetic mutations. The result was the re-categorization and diagnosis of disease

from a system based largely on symptoms and physical manifestations to one more closely defined by molecular signatures.

1.1 Physical Characteristics associated with Dystrophia Myotonica Type 1

Myotonic Dystrophy or dystrophia myotonica type 1 (DM1) stands as an archetypal example of a disease whose identity has been re-examined and redefined in the genetic era. DM1 ranks as one of the most common types of muscular dystrophy and is considered the highest overall contributor to adult muscular dystrophies. It is estimated that as many as 20 in 100,000 people are affected by DM1[1]. The characteristics associated with the syndrome were first identified in the early 1900s by the German physician Hans Steinert[2]. Because of his work, this type of muscular dystrophy was first referred to as "Steinert's disease". Some of the prominent physical manifestations that are characteristic to DM1 include muscle weakness and wasting, myotonia, cardiovascular disorders and cataracts[3].

1.2 Genetic Description

With the rise of genetic testing, a specific genetic anomaly was identified with DM1. Through patient testing, it was found that the symptoms of DM1 were associated with an extended trinucleotide repeat (CTG) within the dystrophia myotonica protein kinase (DMPK) gene. The repeat sequence is located within the 3' untranslated region of exon 15. This repeat sequence is present within all DMPK transcripts and within a phenotypically normal individual it contains up to 50 repeats[4]. Through a process called repeat expansion, additional CTG repeats can be added during the replication preceding meiosis, allowing the repeat section to be increased. The result is that in subsequent generations these repeats can accumulate causing the repeat expansion to become increasingly enlarged. Individuals with fewer than 37 repeats are considered to be free from the risk of affected offspring[5]. The expansion size can range anywhere from hundreds to thousands of repeats. The severity of the disease, however, increases as the numbers of repeats increases. Alleles containing more than 1000 repeats are responsible for the most severe form of the disease, congenital onset DM1, which carries an increased mortality rate[6]. Table 1 gives a succinct listing of the interaction between trinucleotide expansion length and disease expression patterns as defined by the NCBI[7].

[1] Johnson and Heatwole, 2012
[2] Steinberg and Wagner, 2008
[3] Ranum and Day, 2004
[4] Ibid.
[5] Foff et al, 2011
[6] Magana and Cisneros, 2011
[7] Bird, 1999

Table 1: CTG repeat length and DM1 expression[8]		
$(CUG)_n$		
Repeat Size (n)	**Classification**	**Age of Onset**
35 - 49	Normal	N/A
50 - ~150	Mild DM1	20-70 years
~100 - ~ 1000	Classic DM1	10-30 years
> 1000	Congenital	Birth-10 years

DM1 is one of a number of diseases caused by nucleotide repeat expansions. As part of this class of diseases, DM1 takes its place among spinocerebellar ataxias (SCA), fragile-x syndrome (FXS), Freidrich ataxia (FRDA), as well as Huntington disease (HD)[9, 10]

2. Proposed toxicity mechanisms within DM1: A multi-faceted disease

Knowing the particular genetic mutation associated with a disease state is only the beginning of a full understanding that could lead to practical cures. Following the initial identification of the genetic mutation associated with DM1, much of the research focused on nucleotide repeat disorders has involved determining the molecular mechanisms that lead to the symptoms observed in the disease state. DM1 has proved to be one of the prime models used to study the mechanisms of toxicity of genes bearing expanded repeats. Though some pieces are coming together, the full picture of the mechanisms of pathogenesis of DM1 still remains muddled and inexact. In the quest to discover what is occurring within the cells containing the extended repeat, many mechanisms have been hypothesized and investigated.

2.1 Protein Toxicity

Although DMPK expression by the expended repeat may not be affected, the portion of the 3' untranslated region of the DMPK gene which contains the CTG repeats is overlapped by the promoter for another gene, the sine oculis related homeobox 5 (SIX5) gene[11]. Within cells that contain mutant DMPK genes, there is an indication that SIX5 transcription is impaired. As evidence of this, knockout murine models displayed the occurrence of ocular cataracts, a characteristic of DM1. Because of this result, it is thought that loss of SIX5 may play a role in part of the observable symptoms associated with the disease[12].

Recent studies have also identified the potential for the production of a harmful protein product from the extended repeat sequence through a mechanism called Repeat Associated Non-ATG translation

[8] Bird, 1999
[9] Sicot and Gomes-Pereira, 2013
[10] Nalavade et al, 2013
[11] Sicot and Gomes-Pereira, 2013
[12] Belizil et al, 2013

(RAN translation)[13]. Within expanded CAG sequences, translation has been found to occur through non-ATG-initiation mechanisms. Though the DMPK gene is transcribed in the sense direction, the occurrence of bidirectional transcription has been observed in similar expanded trinucleotide repeat diseases such as Huntington disease-like 2 (HDL2), spinocerebellar ataxia type 8 (SCA8), and fragile-x ataxia syndrome (FXTAS)[14]. This bidirectional transcription within mutant DMPK genes would result in transcripts comprised of elongated CAG and CUG sequences capable of RAN translation. When RAN occurs in DM1 transcripts, the reading frame is arranged in all three windows on both transcripts, resulting in five different theoretical long homomeric protein chains: polyglutamine, polyserine, polyalanine, polyleucine, and polycysteine[15]. Within murine neuroblastoma cells, it was found that the presence of these long homomeric protein chains was associated with cellular apoptosis[16]. This newly investigated mechanism holds much potential in understanding the molecular pathogenesis of DM1 and may be an important cause of DM1 symptoms.

2.2 RNA Toxicity

The main focus of DM1 toxicity, however, has been on the role of the mutant RNA transcripts within the disease pathway. The idea of RNA toxicity is based on RNA-gain of function. Gain of function occurs when any molecule assumes a mechanism or action that it does not normally perform. Mutant RNA is particularly susceptible to picking up new functions due to its ability to form diverse secondary structures and its known place in cellular regulation. The precise processes of RNA pathogenesis are areas of much intense research, but three prime mechanisms of RNA sabotage have come into focus: the sequestration of MBNL1, the foci formation of the MBNL1 and mutant DMPK complex, and the overexpression of CUGBP1.

MBNL1 is a member of the musclebind family of proteins and is responsible for the regulation of alternative splicing of specific genes, including the pre-mRNA of the chloride channel (CLCN1), insulin receptor (IR) and cardiac troponin-T (TNNT2) genes.[17, 18] The presence of extended CTG repeat results in the binding of the MBNL1 to the mutant DMPK RNA[19], which recognizes the double stranded hairpin structures formed by the CUG repeats[20]. The MBNL1 and mutant DMPK complex is proposed to affect the cell in two ways. The first and simplest consequence comes from the loss of function of MBNL1.

[13] Belizil et al, 2013
[14] Rnoux andTodd, 2012
[15] Belizil et al, 2013
[16] Zu et al, 2011
[17] Nelson et al, 2013
[18] Nalavade et al, 2013
[19] Ibid.
[20] Cho and Tapscott, 2007

Because of the exclusive binding that occurs between MBNL1 and the mutant DMPK, it is proposed that this sequestration results in MBNL1 being unavailable for the regulation of splicing for which it is responsible[21]. With the mutant DMPK-induced loss of function of MBNL1, a high rate of aberrant splicing is observed among cellular transcripts, many of which are linked directly to phenotypic manifestations of DM1[22]. As evidence of this, transgenic mice containing non-binding MBNL1, display abnormalities and defects that are consistent with those observed in DM1, in particular, the occurrence of myotonia, ocular cataracts and aberrant splicing patterns[23].

CUGBP is another important RNA binding that has been implicated in the molecular pathogenesis of DM1. Like MBNL1, CUGBP is integral to splicing pattern determination and the two proteins work in concert to regulate splicing patterns[24]. CUGBP has also been identified as being important in mediating mRNA decay and increasing translation of proteins such as p21[25,26]. When MBNL1 concentrations are disturbed by the formation of the mutant DMPK-MBNL1 complex and its segregation to the nucleus, CUGBP levels are increased. The abnormal ratio of these two proteins with DM1 cells is similar to that in the embryonic state[27].

The loss of MBNL1 and CUGBP1 function has been shown to be only a partial contributor to DM1 pathogenesis. Studies point to the MBNL1 and mutant DMPK complexes being integral to the development of the DM1 phenotype. Within DM1 cells, the MBNL1-mutant DMPK complexes form foci which are localized in the nucleus. Though healthy cells do not typically display these foci, it was found that the position of these complexes in the nucleus rather than in the cytoplasm was key to DM1 toxicity. Why cytoplasmic foci would not display the same toxicity as nuclear foci is not known, but it gives an important indication that part of the toxicity caused by these complexes must involve interference with the normal processes of the nucleus[28, 29]. It should also be noted that both CAG and CUG repeats have been found to equally contribute in foci formation[30]. These foci are increasingly becoming the most strongly associated mechanism with the pathogenesis of DM1. A summary of proposed mechanisms can be seen in table 2.

[21] Sicot and Gomes-Pereira, 2013
[22] Renoux and Todd, 2012
[23] Kanadia et al, 2003
[24] Ward et al, 2005
[25] Vlasova et al 2008
[26] Timchenko et al, 2004
[27] Sicot and Gomes-Pereira, 2013
[28] Mankodi et al, 2001
[29] Taneja et al 1995
[30] Ho et all, 2005

Table 2: Proposed Mechanisms of Toxicity within DM1					
Protein					
Type	**Category**	**Description**	**Result**	**DM1 Phenotypes Associated**	**Sources**
DMPK protein loss	Loss of function	Mutant DMPK transcripts interfere with normal translation processes	Decreased levels of DMPK protein	Late onset myopathy; delays in cardiac conduction	Jansen, et al, 2003; Berul et al, 1998
RAN translation	Gain of function	Random non ATG initiated translation occurs	Homopolymeric polyglutamine, polyalanine and polyserine proteins created which can interfere with cellular processes	Still under investigation	Cleary, Ranum, 2014; Zu et al 2010
SIX5 gene expression disruption	Decreased expression	Overlap of DMPK UTR with promoter of SIX5 gene	Extended repeats in DMPK may inhibit or limit expression of the SIX5 protein	Ocular cataracts	Flippova, et al, 2001
RNA					
MBNL1 sequestration	Loss of function	MBNL1 associates with extended repeat portion of mutant DMPK	Binding of MBNL1 to DMPK transcripts results in MBNL1 not being available for splicing regulation of normal target proteins	Myotonia, ocular cataracts, cardiac conduction defects	Sicot et al, 2013; Kanadia et al, 2003
MBNL1-DMPK nuclear foci formation	Gain of Function/interruption of baseline	MBNL1 complexes with mutant DMPK and forms foci within nucleus	Foci seem to be toxic when located within the nucleus	Still under investigation	Mankodi et al, 2001; Taneja et al, 1995
CUGBP1 (CELF) upregulation	Over-expression	CELF expression associated with MBNL1, loss of MBNL1 results in increased expression of CUGBP	Missplicing of targeted transcripts including Tnnt2, Mtmr, Clcn1; return to fetal splicing patterns; Insulin resistance	Myotonia, muscle wasting, DM1 histopathy,	Ho et al, 2005; Ward et al, 2010; Timchenko et al, 2001; Philips et al, 1998

3. Methods of Repair of DM1

The understanding of disease processes is driven by the quest to find methods of alleviation and treatment. Increased genetic classification and identification of diseases has given rise to new forms of treatment. Targeting molecular sources of disease is all part of the increasingly prominent field of gene therapy. Traditional gene therapy approaches have mainly focused on introducing complete genes in order to restore proper protein levels within a system. Though this aspect may relieve some symptoms, the previous discussion on mechanisms of disease shows that in a DM1 individual the main pathogenesis is due to the presence of mutant DMPK RNA. Because of this, molecular therapeutic treatments for DM1 must include targeting of these toxic RNA transcripts. Ideally, molecular therapy for correction of DM1 would fulfill all of the following requirements. 1) Therapy mechanism would be specific to mutant DMPK transcripts only. 2) Therapy molecules would be small enough to effectively administer. 3) Therapy would retain the normal transcription levels dictated by the cell and would not interfere with cellular control mechanisms.

Several strategies of targeting extended repeat DMPK transcripts have been proposed to treat DM1. The use of small interfering RNA (siRNA) has been proposed and tested as a possible mechanism for mutant DMPK RNA repair. SiRNA are small double stranded RNA molecules homologous to an intended target. These molecules have been shown to bind to target RNA and induce post transcriptional silencing through targeting of mRNA for degradation by endogenous enzymes. Within a DM1 model, siRNA modified for nuclear localization were shown to successfully degrade nuclear and cytoplasmic DMPK transcripts. The difficulty arising from this method of DM1 alleviation, however, was the failure of the mechanism to distinguish between the mutant and wild-type DMPK transcripts[31]. Additionally, the destruction of DMPK transcripts that occurs in this therapy greatly alters the steady cellular levels of DMPK protein being translated, an event which usually has severe consequences to the cell.

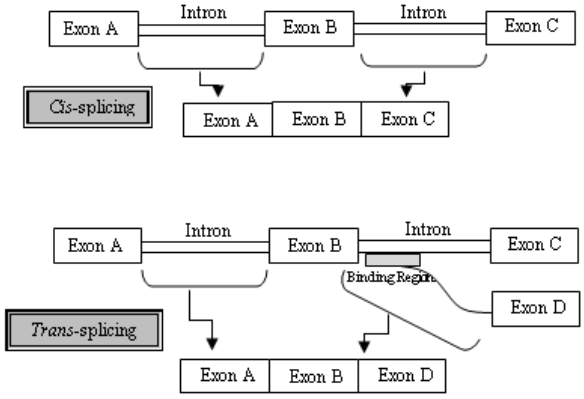
A more promising method is to be found in the family of antisense oligonucleotides (AONs). Two leading classes of these AONs have been used with DM1, 2'O-methyl-modified AONs (MOEs) and phosphodiamidate morpholino antisense molecules (PMOs or morpholinos)[32]. Similar to the use of siRNA, AONs work by post transcriptional gene silencing, either by targeted degradation or by steric interference induced inhibition. Certain MOEs have been tested within a DM1 cell model, and it was found that this therapy model appeared to effectively degrade and reduce the number of mutant DMPK mRNA within both the cytoplasm and the nucleus. In vivo trials showed up to 80% silencing of the

[31] Langlois, et al, 2005 J. Biological Chemistry
[32] Pennock et al, 2011

expanded repeat DMPK transcripts[33]. A study has also shown morpholinos successful in disrupting the mutant DMPK mRNA and MBNL1 foci within the nucleus. There was also some indication that the morpholinos might increase the degradation of these transcripts[34]. In both cases, however design of these AONs was only theorized to be specific to extended CUG repeats. Whether normal DMPK transcripts were also affected was not thoroughly evaluated, though in general the specificity of morpholinos has been shown to be fairly high[35]. Though the use of AONs holds the possibility of disrupting the toxic DMPK foci, as with the previously discussed RNA interference pathways, these therapies fall short of the ideal in their inability to retain the normal DMPK expression patterns that occur when both DMPK alleles are producing functional mRNA.

Answering the call of this short-coming, a new area of gene therapy has risen and begun to take its place: mRNA repair through the use of trans-splicing. Eukaryotic mRNAs require post-transcriptional modification which includes the splicing together of exons and the removal of introns. The splicing normally observed in this stage is referred to as cis splicing, and occurs within a single linear pre-mRNA molecule. The arrangement of exons and exclusion of different areas of the molecule is in part responsible for the great variation seen in eukaryotic organisms. Trans-splicing works similarly, however, the splicing in this instance is no longer within the same molecule, but occurs between two different pieces of RNA. Figure 1 gives a visual example of the difference between the two methods of splicing.

Figure 1



As can be seen in the figure, trans-splicing allows the substitution of another portion of RNA within a modified mRNA transcript. The application to genetic therapy is readily apparent. Different from the methods previously mentioned, trans-splicing could potentially repair mutant transcripts prior to translation and without modification or alteration of the promotion or transcription of the gene. Within DM1, trans-splicing would allow the mutant exon 15, containing the extended trinucleotide repeat, to be replaced with a normal exon 15, containing fewer than 50 repeats.

[33] Mulders et al, 2009
[34] Wheeler et al, 2009
[35] Pennock et al, 2011

The therapeutic use of trans-splicing was first explored with the use of ribozyme-mediated trans-splicing. This type of trans-splicing repair makes use of a synthetically arranged sequence containing both a binding domain for locating of the target RNA, and the exonic sequences to be spliced. In order for splicing to occur within this model, however, a RNA catalyst, or ribozyme must also be included within the therapeutic molecule. Past studies have examined the repair characteristics of this method of therapy in several disorders including their repair efficiency in DM1 fibroblast cells[36]. Though repair has been observed using such mechanisms, ribozyme mediated trans-splicing has several shortcomings which prevent its progression to a clinically relevant therapy option. The most prominent of these are linked to the necessary inclusion of the ribozyme. The main difficulty faced with this approach was the inefficiency of the included ribozyme within physiological Mg+ concentrations[37].

In 1992, it was discovered that mammalian cells possessed the endogenous machinery necessary for performing trans-splicing[38]. This revelation opened a new opportunity for the use of trans-splicing. Within the mammalian cell, the enzyme responsible for splicing is the spliceosome. Based on the finding that eukaryotic spliceosomes were capable of performing trans-splicing as well as the usually observed cis-splicing, a novel method of RNA repair was introduced: spliceosome mediated trans-splicing (SMaRT). Since all eukaryotic cells contain the spliceosome needed for SMaRT technology, the need to supply machinery such as ribozymes for initiating the splicing event was obviated. Since the strongest setbacks in feasibility of use of trans-splicing for therapy were due to presence of the ribozymes, SMaRT has become a much more promising type of RNA repair.

Since its introduction, the mRNA repair capabilities of SMaRT therapy have been examined in a growing number of genetic disorders. Artificial trans-splicing has been conducted in disease models of hypertrophic cardiomyopathy[39], Huntington's disease (HD)[40], epidermolysis bullosa simplex with muscular dystrophy (EBS-MD)[41], and hemophilia A[42]. This type of trans-splicing was also used within the context of DM1 by Chen et al in 2009. Most of these studies have served as proof of concept for SMaRT mRNA repair.

The translational potential of SMaRT technology is difficult to judge due to several reasons. One reason is due to the wide variability in efficiency measurements which prevents direct comparison. Several *in*

[36] Phylactou, et al, 1998
[37] Wood, et al 2007
[38] Bruzik and Maniatis, 1992
[39] Mearini et al, 2013
[40] Rindt 2012
[41] Wally et al, 2007
[42] Chao et al, 2003

vitro experiments have reported trans-splicing efficiency between 1-14%. *In vivo* studies, however, the efficiency has been found between 3-7%[43]. In a study where SMaRT was applied directly to DM1, the efficiency of repair was 1.8-7.41%[44].

Additionally, the differences in disease models also effects the conclusions as to the efficiency of the SMaRT therapy. Many of the disease models used thus far have been made by the use of artificial constructs, where the desired target of repair is found within an extra-chromosomal plasmid. For some of the genetic mutations, such as HD and EBS-MD, the trans-splicing evaluation of the PTMs was also evaluated within patient-derived cell lines endogenously containing the mutated alleles[4546]. In general, the trans-splicing efficiency was greatly decreased within most of the more clinically relevant conditions. The efficiency of splicing repair in these studies was much less than in the artificial constructs.

Despite the diversity of these studies, a common theme has been emphasized: the need for more efficiency and specificity of the splicing events. For diseases where loss of protein is the main influence in disease prognosis, small levels of trans-splicing repair may be sufficient to alleviate symptoms. Because if this, PTMs with low splicing efficiency may be satisfactory. It is important to note however, that low levels of trans-splicing repair may be enough to alleviate the disease state. Even with the low efficiency of current SMaRT technology, research using murine disease models have shown the technology capable of causing significant phenotypic changes in hemophilia A and hypertrophic cardiomyopathy[4748].

For DM1, however, the presence of mutant mRNA is the main source of toxicity. It is not known how many mutated DMPK transcripts are required for toxicity to occur. Thus it can be assumed that DM1 will require much higher trans-splining efficiency due to the RNA toxicity nature of the disease. Before SMaRT can become a feasible clinical therapy option for patients suffering from DM1, it is imperative that the splicing efficiency of the therapeutic PTMs be improved. Additionally, a clinically relevant model of DM1 has not yet been used to evaluate SMaRT technology. Since the efficiency of trans-splicing is not adequately reflected in artificial disease constructs, any improvement in efficiency must be evaluated within a model that reflects the complexity of the actual disease state.

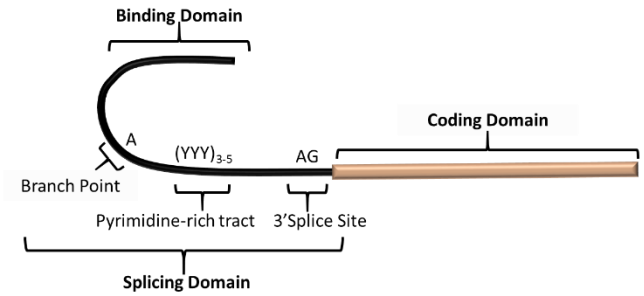
[43] Mansfield et al, 2004
[44] Chen et al, 2009
[45] Rindt, 2012
[46] Wally et al 2007
[47] Chao et al 2003
[48] Mearini et al, 2013

4. Experimental Details

The purpose of this study was to improve PTM efficiency and specificity and to examine the trans-splicing repair of PTMs using a patient-derived DM1 cell. Because SMaRT technology relies on endogenous cellular mechanisms for splicing, the key to improving trans-splicing efficiency is to improve the PTM design so that it can be correctly identified by the cell and positioned near the desired target transcript. Understanding how to improve PTM efficiency requires knowledge of PTM design as well as an understanding of how genetic material is naturally positioned and associated within the nucleus.

4.1 Experimental Theory

Figure 2: Basic PTM Structure



Basic PTMs are comprised of several specific sequences that fall into three main categories based on their function: the binding domain, the splicing domain, and the coding domain[49]. Each serves an important function within the cell and are visually represented in figure 2.

The coding domain contains the portion of RNA that is to be incorporated into the target transcript. The splicing domain consists of several unique features, most importantly, the pyrimidine-rich tract, the 3'splice site and the branch point. These are all features which allow the spliceosome machinery to identify this molecule as a splicing candidate and to correctly associate with it. Typically, the splicing domain portions of the PTM are analogous to the splicing factors that would be found within an endogenous intron. The final sequence of interest is the binding domain. The purpose of the binding domain is to provide a sequence which will cause the cell to position the PTM near its target gene. Because splicing occurs co-transcriptionally, it is necessary that the PTM be located near to the actively transcribed target gene. It is this portion which largely determines the molecule's specificity and efficiency. The best PTM design is one which contains a binding domain that has learned to speak the language of nuclear positioning and sends a clear address of destination.

[49] Mansfield et al, 2004

Since the goal is to localize the PTM to the position in the nucleus where transcription of the desired target is occurring, an understanding of nuclear organization is necessary. The idea that the nucleus is a random network of genes and proteins has long been refuted and it is now known that the structure of its interior is highly complex. Where genetic material is positioned within the nuclear matter is closely linked to the regulation of gene expression. It has been found that within the nucleus, transcription tends to occur within foci, or in distinct groupings commonly referred to as transcription factories. These transcription factories contain several genes which are actively transcribed by RNA polymerase II[50]. What is of importance is how the grouping of genes are determined within the factories. To date, this area currently under investigation, however it has been shown that homology of genes is a strong influence in the grouping seen in these foci[51]. This concept was promoted by the finding that when a plasmid containing a β-globin gene was introduced to a mammalian cell, the nuclear machinery colocalized the plasmid with the homologous endogenous gene[52]. Other studies have indicated that the promoter region of the gene may be the sequence responsible for inclusion in the transcription factory, that is, that homologous promoters are sequestered into the nuclear foci[53].

In order for a PTM to be efficiently transcribed and trans-spliced into the correct target, it must be located within the same transcription factory as the gene of interest. Traditionally, the binding domain of PTMs has consisted of a sequence that is antisense to an intronic portion of the targeted pre-mRNA. This design is founded on the idea that the main stimulus in nuclear positioning is canonical Watson-Crick base pair complementarity. The recent investigations into the organization of the nucleus and the transcription factories cited above challenge this assumption. Based on the previously stated study regarding plasmid positioning within the genome, there is strong evidence that homology rather than complementarity may provide the strongest nuclear positioning "address". Because of this it is proposed that using a PTM containing a binding domain homologous to the target will increase the efficiency of trans-splicing.

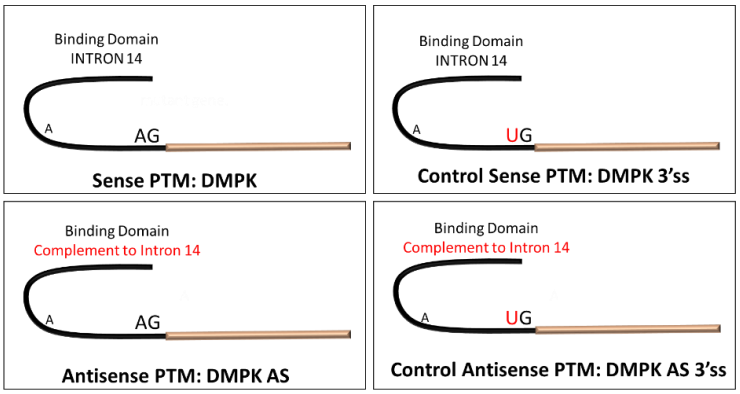
Experimental Design

In this experiment, PTMs containing binding domains which were homologous to intron 14 of the DMPK gene were created. The efficiency of these molecules was compared with that of PTMs containing the traditional antisense binding domain. The efficiency of the two was evaluated by the use of two control PTMs. For each of the experimental PTM types, controls were created by alteration of

[50] Chakalva and Fraser, 2010
[51] Binnie et al, 2006
[52] Ashe et al, 1997
[53] Larkin et al, 2013

the 3' splice site needed for recognition of the spliceosome. Figure 3 shows the four experimental PTMs created for the experiment. An overall control was also used where no PTM was added to the cell culture.

Figure 3: Experimental PTMs Created



The model used for this experiment was a GM23300 lymphocyte cell line derived from a patient suffering from DM1 and acquired from Coriell repositories. This cell line contained both a mutant DMPK allele with around 150 to 160 CTG repeats present in the mutant gene as well as normal allele. The large difference in size between the wild type and the toxic DMPK transcript allows the two to be separated by size. Thus, when run on a gel, a sample containing both types of transcripts would be expected to show a high band around 990 bp and a low band around 500 bp.

Evaluation of the presence of mutant DMPK transcripts by band density was the method employed for this experiment. The density of the high band within the gel indicates the amount of toxic transcripts. Because a normal wild-type transcript is already present within the cell, the low band contains both trans-spliced correct DMPK mRNA as well as the normal transcripts derived from the endogenous wild-type allele and prevents the direct determination of repaired transcripts. However, as the efficiency of PTMs increases, the density of the high band should decrease and the low band increase. Using this ratio of the two bands, the efficiency of the PTMs of interest can still be determined. To standardize the expression levels between samples the amount of the housekeeping gene GAPDH was quantified.

METHODS AND MATERIALS

1. *Designing PTMs*

1.1 Replication of normal intron 14 and exon 15

A cell line containing two normal DMPK alleles (<50 CTG repeats) was used to isolate the coding domain for the PTMs. The DNA from these cells was gathered using DNeasy DNA extraction kit from Qiagen. PCR was performed with the extracted DNA as template using forward and reverse PCR

Primers, DMPK P1 and DMPK P2. These primers were designed to amplify the portion of the gene containing intron 14 and exon 15. Table 3 provides a tabulated compilation of all primers used in the experiment. An inserted CACC sequence was added at the beginning of the forward primer, DMPK P1 to allow for insertion into the TOPO vector. AcuPrime GC Rich DNA polymerase was used due to the high CG content of the desired sequence.

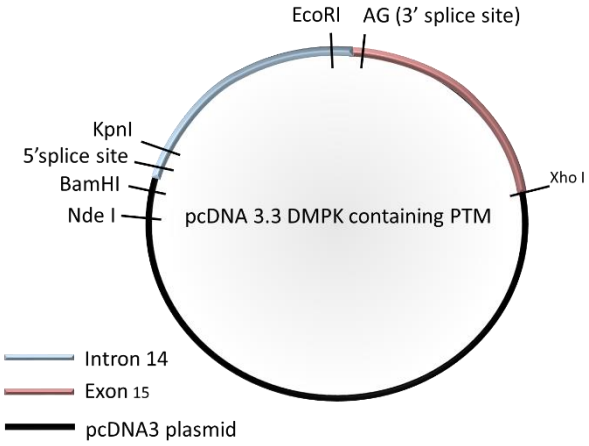
The PCR reaction was performed using 200 ng of template DNA. The resulting product was run on a 0.5% agarose gel alongside a 1 kb ladder. A visible band appeared within the range of the expected size for the PCR product, which was approximately 1356 bp. The portion of the gel containing this product was excised and the genetic material removed from the agarose with a gel extraction kit.

1.2 Creation of sense PTM plasmids: pc3.3 DMPK

Using the pc.DNA3.1 Directional TOPO Expression kit from Invitrogen, the PCR product was ligated into the backbone according to the manufacturer's protocol. These plasmids were introduced into chemically competent TOP10 bacterial cells via heat shock, plated on a pre-warmed plate containing ampicillin and allowed to grow overnight. It was observed that the growth of these colonies was much slower than to be expected.

Three colonies were chosen from the plate and the plasmids were extracted using Qiagen's MiniPrep kit. To confirm the presence of the DMPK portion within the cellular plasmids, the collected genetic product from the three cell lines were treated with Nde I and XhoI restriction enzymes. After separating the fragments by gel electrophoresis, the second sample was the only one to show a band within the proper range for the desired product. This clone was submitted for sequencing which confirmed that the topo plasmid contained the amplified intron 14 and exon 15.

Figure 4: pcDNA diagram



Further modifications were made to the inserted DMPK portion. Using site directed mutagenesis the 5' GT splice site was removed to prevent any cis-splicing from occurring within the molecule. An EcoRI site was also added just upstream of exon 15. An image of the modified pcDNA plasmid can be seen in figure 4.

Table 3: PCR Primers		
	Sequence (5'→ 3')	
	Forward	**Reverse**
DMPK P1	CACCTACGTCCGGCCCAG G	
DMPK P2		TAGCTCCCAGACCTTCG
DMPK3ss_for	CGCCCTCTCCCGCACGTCCCTAGGC	
DMPK3ss_rev		GCCAGGCCTAGGGACGTGCGGGGAG
DMPKin14 P1	AACTTGGTACCCCCGGCATGGGCCT	
DMPKin14 P2		TCAACAGAATTCGAGCTCGGATCCAGT
DMPK RT 3	CGGATCCTTCCCATCTA	
DMPK RT 4		CTGGCCGAAAGAAAGAAATG

1.3 Creation of anti-sense binding domain PTM: pc 3.3 DMPK AS

To create an anti-sense sequence, the binding domain was amplified by PCR using primers DMPKin14 P1 and 2. These were designed to introduce a KpnI restriction site at the end of the binding domain of intron 14 and an EcoRI restriction site upstream of the donor splice site. Sense PTM plasmids and the purified PCR product from this step were both digested with KpnI and EcoRI and then ligated together. Because of the location of the KpnI and EcoRI within the vector, this step reversed the orientation of the binding domain portion, resulting in the desired anti-sense PTM.

1.4 Creation of control PTM plasmids: pc3.3 DMPK 3'ss and DMPK AS 3'ss

Controls were created for both the DMPK and DMPK AS PTMs by alteration of the AG 3' acceptor splice site. Through site directed mutagenesis, the AG splice site within intron 14 was replaced with a theoretically, inoperable AC sequence. This step was performed by the use of primers DMPK 3ss_for and 3ss_rev.

2. Optimization of analysis conditions

2.1. Optimization of PCR reaction

The GM23300 cell-line was the model used for this experiment. Prior to any experimentation, the baseline parameters of the experimental conditions were determined by optimization of RNA extraction, RT-PCR and electrophoresis conditions.

Total mRNA from the DM1 cells was extracted using RNeasy mini kit from Qiagen. This mRNA product was subjected to RT-PCR with using primers DMPK RT P3 and P4. These primers were specialized to amplify the portions of transcript that included both the exon 14-15 junction and the $(CTG)_n$ repeat in Exon 15. Transcripts from the mutant allele were expected to be around 884-914 base pairs with the normal allele expressing mRNA of only 494 bp in length. These two transcripts were visibly distinguishable when run on a 0.5% agarose gel.

Initially, some trouble was met in the PCR reaction particularly in the amplification of the mutant DMPK transcripts due to the high GC content. This difficulty was removed by the use of 5% DMSO in the PCR reaction mixture. The resulting optimized PCR mixture and conditions for the DMPK replication is listed in Table 4. GAPDH was used as the housekeeping gene. The PCR reaction analysis of this product was also adjusted prior to experimentation. Table 5 gives the subsequent settings for RT-PCR of GAPDH. It should be noted that DMSO was not needed for the GAPDH analysis.

2.2 Optimization of Electrophoresis

The DMPK was found to separate best when run on a 1% agarose gel for an hour and 20 minutes. GAPDH, however, only required the length of an hour for electrophoresis. These conditions were kept standard throughout the experiment.

3. PTM efficiency experiment

3.1 Lipofectamine transfection of PTMs into DM1 cell line

The GM233300 cells were thawed and allowed to grow in RPMI media supplemented with 15% FBS and 1% penicillin-streptomycin. Cell density was determined by hemocytometer-based counting and the culture was resuspended at a concentration of $1.2x10^6$ cells per milliliter.

Cells were plated in a 24 well plate. Each well was seeded with 0.5 milliliters of the DM1 cell culture. Five different conditions were analyzed in triplicate. Two experimental PTMs were used, DMPK and DMPK AS, alongside the two control PTMs, DMPK Δ3'ss and DMPK AS Δ3'ss and the final condition contained no PTM. Since each of the four PTM solutions had differing levels of genetic material, serum free medium was added individually to bring the concentration of each to 1.2 micrograms of DNA per 50 microliters. Following the protocol for lipofectamine transfection, the lipofectamine was diluted with medium and combined with each of the PTM solution in a 50:50 ratio. After allowing this mixture to incubate, 100 microliters of each lipofectamine and PTM solution were added to each well, respective of their identity. Cells were then incubated for 24 hours at 37 C.

3.2 RNA extraction and PCR assembly

Following incubation, the cells in each well were removed. After pelleting by centrifugation, the cells were lysed using QIAshredder and RNA extracted using RNeasy mini kit. The extracted RNA was spectroscopically quantified to determine concentration.

Two RT PCR reactions were assembled for each of the reactions: one using DMPK primers and the other GAPDH primers. The PCR reactions were mixed and run according to the optimized conditions listed in Table 4 for the DMPK reaction and Table 5 for the GAPDH reaction. Because of the varying concentrations of base RNA, the volume added of each was determined so that a total of 100 nanograms of RNA was added to each reaction. The difference in volume for each was compensated by the addition of sterile water.

Table 4: RT-PCR DMPK Conditions

5% DMSO Reaction			**PCR DMPK Schedule**		
	Quantity (λ)			Time	
2x Reaction Mixture	25		55 C	30 minutes	
DMPK RT P3	1		94 C	2 minutes	
DMPK RT P4	1		94 C	15 seconds	Repeat 39x
SIII RT	2		62.9 C	15 seconds	
DMSO	2.5		68 C	75 seconds	
RNA	Volume= 100ng		68 C	5 minutes	
PCR H_2O	Adjusted to bring total volume to 50 λ		4 C	∞	
Total	50				

Table 5: RT-PCR GAPDH Conditions

Reaction			**PCR DMPK Schedule**		
	Quantity (λ)			Time	
2x Reaction Mixture	25		55 C	30 minutes	
GAPDH rev	1		94 C	2 minutes	
GAPDH for	1		94 C	15 seconds	Repeat 39x
SIII RT	2		53.6 C	15 seconds	
RNA	Volume= 100ng		68 C	75 seconds	
PCR H_2O	Adjusted to bring total volume to 50 λ		68 C	5 minutes	
Total	50		4 C	∞	

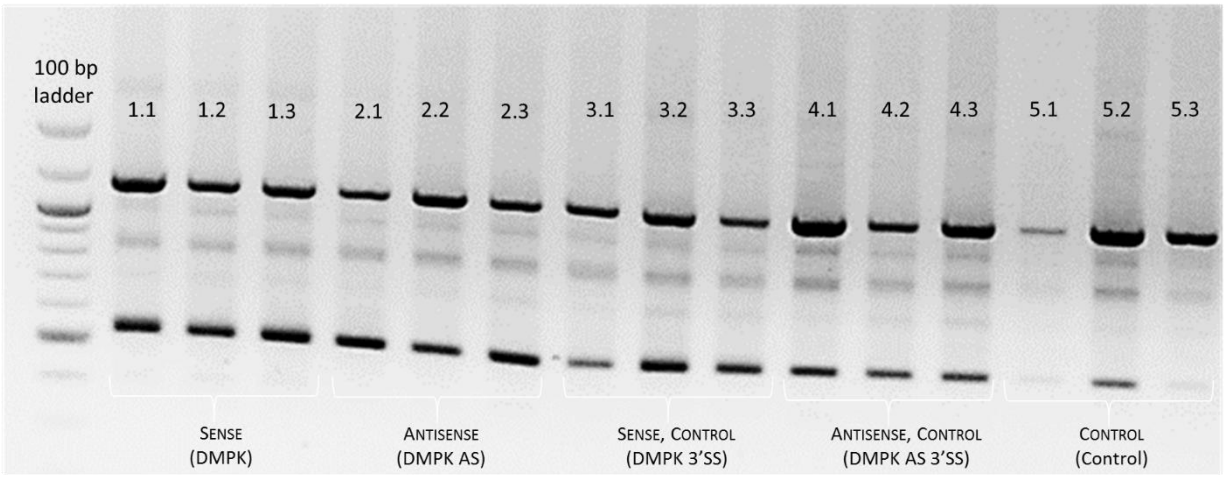
3.3 RNA Analysis

The resulting PCR products were run on the standard 1% agarose gel for the times determined in 2.2. The samples were run alongside a 100 bp ladder in a 16 well gel. Using GeneSys software, the densities of each of the bands was determined and each normalized with the results of the GAPDH expression gel. The ratios between the mutant and the normal DMPK transcripts were compared. The efficiency, that is, the ratio of normal transcripts to mutant, of each PTM was compared with each other and with the controls. Figure 6 contains the results of the DMPK gel and the numerical values for each can be found in table 4.

RESULTS

The gel results showing the mRNA DMPK products for each sample can be seen in Figure 5. From this gel it can be seen that all the samples displayed a distinct high band and low band corresponding to mutant DMPK mRNA and wild type/corrected DMPK mRNA as expected. The empty control samples seemed to show a much less distinct wild type sized product. Table 6, however gives the numerical results for densities as determined by GeneSys. Figure 6 gives ratio of densities of the high band over the low band, that is, the number of mutant transcripts per normal transcripts for each experimental condition after each was standardized by GAPDH expression.

Figure 5: experimental DMPK products



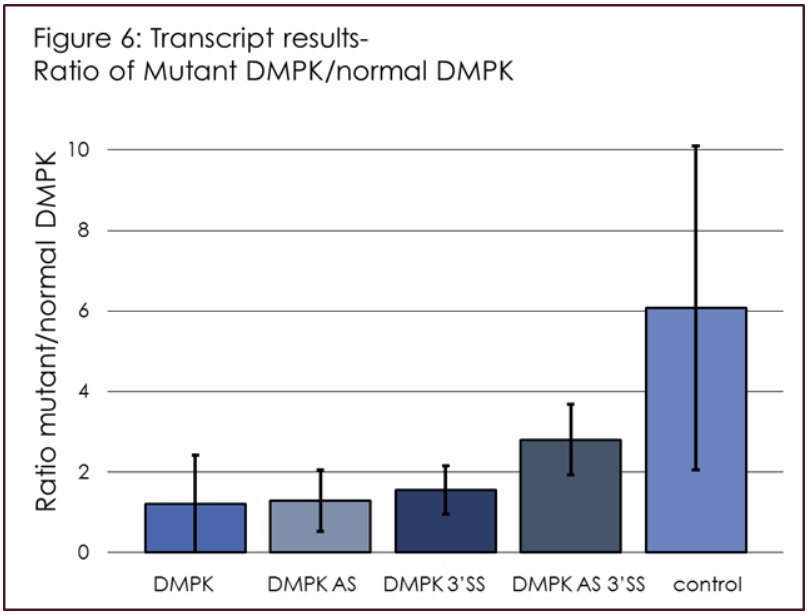


Table 6: Results- DMPK RT-PCR product band density evaluation

	DMPK		GAPDH		DMPK/GAPDH		Hi/Lo	Average	Standard Deviation
	~1100bp Hi (Mutant)	~500 bp Lo (correct)			Hi	Lo			
1.1	218.1097	146.62	992.3121		0.219799	0.147756	**1.487585**	**1.207181**	**0.253095**
1.2	142.5281	125.2107	1093.682		0.13032	0.114485	**1.138306**		
1.3	166.5916	167.3189	1116.483		0.149211	0.149862	**0.995653**		
2.1	112.2278	154.0862	170.0726		0.659882	0.906002	**0.728344**	**1.286695**	**0.771907**
2.2	211.6267	97.63383	1345.464		0.157289	0.072565	**2.167555**		
2.3	139.2424	144.4144	1273.33		0.109353	0.113415	**0.964186**		
3.1	139.653	62.85316	1257.736		0.111035	0.049973	**2.221893**	**1.551194**	**0.596997**
3.2	183.4277	135.4922	1157.47		0.158473	0.117059	**1.353788**		
3.3	104.6203	97.05919	1877.922		0.055711	0.051684	**1.077902**		
4.1	323.1386	91.56604	1162.54		0.277959	0.078764	**3.529022**	**2.803667**	**0.879389**
4.2	127.285	69.72279	1119.243		0.113724	0.062295	**1.825587**		
4.3	227.469	74.42402	1075.686		0.211464	0.069187	**3.056392**		
5.1	44.55943	17.83333	1004.617		0.044355	0.017751	**2.49866**	**6.07808**	**4.021466**
5.2	306.2594	57.71995	914.3212		0.334958	0.063129	**5.305954**		
5.3	196.0608	18.79845	837.5276		0.234095	0.022445	**10.42963**		

Key: 1) DMPK 2) DMPK AS 3) DMPK Δ3'ss 4) DMPK AS Δ3'ss 5) Control

DISCUSSION

A visual analysis of the results as seen in figure 6 may at first glance present a confusing picture. Initially, it gives the indication that the ratio of mutant to normal transcripts was greater in the overall control, and that the presence of PTMs regardless of their splicing capabilities seemed to decrease the amount of toxic transcripts. The wide error bars seen in the graph, however, give an indication that the numerical results might be lacking in significance ANOVA and post hoc test analysis confirmed the fact that the gathered data showed not statistical difference between any of the five experimental groups. The results, therefore show no difference in mutant and normal DMPK transcripts between the cells containing either type of PTM and the three controls.

The lack of difference between the groups can be interpreted in two ways: either no trans-splicing repair of DMPK occurred within the model or the trans-splicing repair of DMPK was too minimal to detect. Taking the first interpretation to be true, one is left to answer why no SMaRT repair occurred. Some possible scenarios include: 1) the PTM never reached the nucleus 2) the PTM never reached the target transcription factory.

The possibility of the PTM failing to reach the nucleus the least likely cause. Both lipofectamine, the transfection regent used for introduction of the PTM into the cell, and the pc3.1DNA vector used to transport the PTM sequence are standard laboratory tools. Because of these facts, the chance that the PTM plasmid failed to reach the nucleus is fairly slim and does not provide a reasonable account of the results. All the same, the possibility that some contaminant may have destroyed the PTM culture or that the PTMs were degraded in the process of storage is always a lingering possibility, albeit unlikely.

A more worthwhile postulate is that no trans-splicing occurred due to failure of the PTM to be included in the transcription factory containing the DMPK gene. The failing of PTMs to find their proper position in the transcription factory has been discussed before. In this case, it is not likely to be wholly due to faulty binding domain. PTMs of the same construction as the experimental antisense PTMs have been used successfully in the past. If the problem was due to the binding domain, it would be expected that at least a small level of splicing would have been observed in the culture receiving the DMPK AS plasmid. However, neither experimental culture showed splicing. This seems to indicate that the targeting provided by the binding domain did not play a role in where the experimental PTMs were positioned within the nucleus.

In this experiment, a fairly high concentration of plasmids was used, 1.2 micrograms per 600,000 cells. This over dosage of plasmids had been intentional to ensure that PTMs would successfully reach all of the cells. However, how the number of plasmids affects the sorting processes of the nucleus is not well

known. It is possible that only plasmids under a certain concentration will be integrated into endogenous transcription factories. If that concentration is exceeded, it is possible that the nuclear mechanisms instead associate the plasmids with each other resulting in the creation of an artificial transcription factory. The high quantity of PTM plasmids within the nucleus of the experimental cultures may have resulted in them being sequestered into specialized plasmid foci, rather than integrating them into the DMPK transcription factory. The result of such would be that no splicing would occur and no change would be seen in the number of mutant DMPK transcripts when compared to their normal companions, a result that matches the experimental data.

It is additionally worth considering the possibility that trans-splicing of mutant DMPK did occur, but at low enough levels that the event was undetected. This points to a slight disadvantage of the current method of data evaluation. Because DMPK transcripts are evaluated by length, the presence of the normal allele prevents direct detection of trans-spliced products. The failure to differentiate between the two transcripts is a drawback that should be remedied in future investigations and is required for understanding what errors are occurring within the experiment.

Though the results of this experiment failed to address the hypothesis and to determine whether the efficiency SMaRT technology could be improved by a use of a sense binding domain in the PTM, it has provided valuable ground work for future investigations. Further work should focus on two aspects: improving differentiation between normal and trans-spliced DMPK transcripts and evaluation of the effects of plasmid density upon PTM incorporation and segregation within the nucleus.

One method that would allow distinction to be made between normal and repaired DMPK transcripts would be to include a unique sequence within the coding region of the PTM which would allow the isolated detection of trans-spliced products. This has the drawback of potentially requiring two PCR reactions to be run, one looking at endogenous transcripts and the other at repaired products. A different approach could be prepare the PTM to contain a repeat sequence that was of intermediate length, perhaps containing 50-100 additional repeats. The same technique of analysis presented in this experiment might be used. The result would be that instead of two bands as observed, three bands should occur: the high band for the toxic transcripts, the low band for the endogenous normal transcripts, and an additional third band somewhere between the two for trans-spliced products. The advantages of such an approach would be that it would allow a direct visualization of all types of DMPK mRNA present within the cell. This method would, however, necessitate that the separation of the three bands to be clear enough for analysis, something that was slightly challenging even when working with two expected bands.

Additionally, future studies should use lower concentrations of plasmids when transfecting cells. To determine whether the splicing is affected by concentration, experiments should be conducted using antisense binding domain plasmids since these have prior experimental evidence of trans-splicing repair. As the concentration of the PTMs is decreased, the trans-splicing rates should increase. This will allow the determination of the most favorable plasmid concentration to be used for further experimentation. Once the plasmid concentration has been optimized, it will be possible to examine the effects on efficiency that are caused by the use of PTMs with sense binding domain.

SMaRT technology holds great promise in the field of gene therapy, specifically in its potential for correcting RNA toxicity diseases such as DM1. Demonstrating its potential, however, still requires much further investigation, particularly in improving the efficiency of the artificial PTM molecules. Though this study was unable to provide proof of concept for the use of sense binding domains, it has been an integral part of the future studies that must be done in order to make SMaRT repair of DM1 a reality. This research has shown the direction that future experimentation ought to take and has set the foundation for further investigation in binding domain improvement.

REFERENCES

Ashe HJ, Monks J, Wijgerde M, Fraser P, Proudfoot NJ. Intergenic transcription and transinduction of the human β-globin locus. *Gene Dev* 1997; **11:** 2494-2509

Belzil VV, Gendron TF, Petrucelli L. RNA-mediated toxicity in neurodegenerative disease. *Mol Cell Neurosci* 2013; **56:** 406-419

Berul CI, Maguire CT, Aronovitz MJ, Greenwood J, Miller C, Gehrmann J, Houseman D, Mencelsohn ME, Reddy S. DMPK dosage alterations result in atrioventricular conduction abnormalities in a mouse myotonic dystrophy model. *J Clin Invest* 1999; **103:** R1-R7

Binnie A, Castelo-Brano P, Monks J, Proudfoot NJ. Homologous gene sequences mediate transcription-domain formation. *J Cell Sci* 2006; **119:** 3876-3887

Bird T. Myotonic Dystrophy Type 1. Gene Reviews. 1993; [Internet]. Accessed Sept 28 2014. http://www.ncbi.nlm.nih.gov/books/NBK1165/

Bitterman PB, Gourdon G, Swanson MS, Moseley M, Ranum PW. Non ATG-initiated translation directed by 5' microsatellite expansions. *PNAS* 2011; **108:** 260-265Bruzik JP, Maniatis T. Spliced leader RNAs from lower eukaryotes are trans-spliced in mammalian cells. *Nature* 1992; **360:** 692-695

Chakalova L, Fraser P. Organization of transcription. *Cold Spring Harb Perspect Biol* 2010; **2:**a000729

Chao H, Mansfield SG, Bartel RC, Hiriyanna S, Mitchell LG, Garcia-Blanco MA, Walsh CE. Phenotype correction of hemophilia A mice by spliceosome-mediated RNA trans-splicing. *Nat Med* 2003; **9:** 1015-1019

Chen HY, Kathirvel P, Yee WC, Lai PS. Correction of dystrophia myotonica type 1 pre-mRNA transcripts by artificial trans-splicing. *Gene Ther* 2009; **16:**211-217

Cho DH, Tapscott SJ. Myotonic dystrophy: emerging mechanisms for DM1 and DM2. *Biochim Biophys Acta* 2007; **1772:** 195-204

Cleary JD, Ranum LPW. Repeat associated non-ATG (RAN) translation: new starts in microsatellite expansion disorders. *Curr Opin Genet Dev* 2014; **26:** 6-15

Flippova GN, Thienes CP, Pen BH, Cho DH, Hu YJ, Moore JM, Klessert TR, Lobanekov W, Tapscott SJ. CTCF-binding sites flank CTG/CAG repeats and forma methylation-sensitive insulator at the DM1 locus. *Nat Genet* 2001; **28:** 335-343

Ho TH, Bundman D, Armstrong DL, Cooper TA. Transgenic mice expressing CUG-BP1 reproduce splicing mis-regulation observed in myotonic dystrophy. *Hum Mol Genet* 2005; **14:** 1539-1547

Jansen G, Groenen PJTA, Bächner D, Jap PHK, Coerwinkel M, Oerlemans F, van den Brock W, Gohlsch B, Pette D, Plomp JJ, Molenaar PC, Nederhoff MGJ, van Echteld CJA, Dekker M, Berns A, Hameister H, Wieringa B. Abnormal myotonic dystrophy protein kinase levels produce only mild myopathy in mice. *Nat Genet* 1996; **13:** 316-324

Johnson NE, Heatwole CR. Myotonic dystrophy: from bench to bedside. *Semin Neurol* 2012; **32**: 246-254

Kanadia RN, Johnstone KA, Mankodi A, Lungu C, Thornton CA, Esson D, Timmers AM, Hausworth WW, Swanson MS. A musclebind knockout model for myotonic dystrophy. *Science* 2003; **302:** 1978-1980

Langlois M, Boniface C, Wang G, Alluin J, Slavaterra PM, Puymirat J, Rossi JJ, Lee SN. Cytoplasmic and nuclear retained DMPK mRNAs are targets for RNA interference in myotonic dystrophy cells. *J Biol Chem* 2005; **280:** 16949-16954

Larkin JD, Papantonis A, Cook PR. Promoter type influences transcriptional topography by targeting genes to distinct nucleoplasmic sites. *J Cell Sci* 2013; **126:** 2052-2059

Magaña JJ, Cisneros B. Perspectives on gene therapy in myotonic dystrophy type 1. *J Neurosci Res* 2011; **89:** 275-85

Mankodi A, Urbinati CR, Yuan Q, Moxley RT, Sansone V, Krym M, Henderson D, Schalling M, Swanson MS, Thornton CA. Musclebind localizes to nuclear foci of aberrant RNA in myotonic dystrophy types 1 and 2. *Hum Mol Genet* 2001; **10:** 2165-2170

Mansfield SG, Chao H, Walsh CE. RNA repair using spliceosome-mediated RNA trans-splicing. *Trends Mol Med* 2004; **10:** 263-268

Mearini G, Stimpel D, Krämer E, Geertz B, Baren I, Gedicke-Hornung C, Précigout G, Müller HA, Eschenhagen T, Voit T, Garcia L, Lorain S, Carrier L. Repair of Mybpc3 mRNA by 5'-trans-splicing in a mouse model of hypertrophic cardiomyopathy. *Mol Ther* 2013; **2:** e102

Mulders SAM, van den Broek WJAA, Wheeler TM, Croes HJE, van Kuik-Romeijn P, de Kimpe SJ, Furling D, Plantenberg GJ, Gourdon G, Thornton CA, Wieringa B, Wansink DG. Triplet-repeat oligonucleotide-mediated reversal of RNA toxicity in myotonic dystrophy. *PNAS* 2009; **106:** 13915-13920

Nalavade R, Griesche N, Ryan DP, Hildebrand S, Krauß S. Mechanisms of RNA-induced toxicity in CAG repeat disorders. *Cell Death Disea* 2013; **4:** e792

Nelson DL, Orr HT, Warren ST. The unstable repeats –three evolving faces of neurological disease. *Neuron* 2013; **77:** 825-842

Pennock Foff E, Mahadevan MS. Theraputics development in myotonic dystrophy type 1. *Muscle Nerve* 2011; **44:** 160-169

Philips AV, Timchenko LT, Cooper TA. Disruption of splicing regulated by a CUG=binding protein in myotonic dystrophy. *Science* 1998; **280:** 737-741

Phylactou LA, Darrah C, Wood MJA. Ribozyme-mediated trans-splicing of a trinucleotide repeat. *Nat Genet* 1998; **18:** 378-381

Ranum LPW, Day JW. Myotonic Dystrophy: RNA pathogenesis comes into focus. *Am J Hum Gen* 2004; **74:** 793-804

Renoux AJ, Todd PK. Neurodegeneration the RNA way. *Prog Neurobiol* 2012; **97:** 173-189

Rindt H. Replacement of huntingtin exon 1 by trans-splicing. *Cell Mol Life Sci* 2012; **69:** 4191-4204

Sicot G, Gomes-Pereira M. RNA toxicity in human disease and animal models: from the uncovering of new mechanism to the development of promising therapies. *Biochim Biophys Acta* 2013; **1832**: 1390-1409

Steinberg H, Wagner A. [Hans Steinert: 100 years of muscular dystrophy] *Nervenarzt* 2008; **79**: 965-970

Taneja KL, McCurrach M, Schalling M, Houseman D, Singer RH. Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues. *J Cell Biol* 1995; **128:** 995-1002

Timchenko NA, Iakova P, Cai Z, Smith JR, Timchenko LT. Molecular basis for impaired muscle differentiation in myotonic dystrophy. *Mol Cell Biol* 2001; **21:** 6927-6938

Timchenko NA, Patel R, Iakova P, Cai Z, Quan L, Timchenko LT. Overexpression of CUG triplet repeat-binding protein, CUGBP1, in mice inhibits myogenesis. *J Biol Chem* 2004; **279:** 13129-13139

Vlasova IA, Tahoe NM, Fan D, Larsson O, Rattenbacher B, SternJohn JR, Vasdewani J, Karypis G, Reilly CS, Bitterman PB, Bohianen PR. Conserved GU-rich elements mediate mRNA decay by binding to CUG-binding protein 1. *Mol Cell* 2008; **29:** 263-270

Wally V, Klausegger A, Koller U, Lochmüller H, Krause S, Wiche G, Mitchell LG, Hitner H, Bauer JW. 5' trans-splicing repair of the PLEC1 gene. *J Invest Dermatol* 2008; **128:** 568-574

Ward AJ, Rimer M, Killian JM, Dowling JJ, Cooper TA. CUGBP1 overexpression in mouse skeletal muscle reporcudes features of myotonic dystrophy type 1. *Hum Mol Genet* 2010; **19:** 3614-3622

Wheeler TM, Sonczak K, Lueck JD, Osborne RJ, Lin X, Dirksen RT, Thornton CA. Reversal of RNA-dominance by displacement of protein sequestered on triplet repeat RNA. *Science* 2009; **325:** 336-339

Wood MJA, Yin H, McClorey G. Modulating the expression of disease genes with RNA-based therapy. *PLoS Genet* 2007; **3:** 845-854

Zu T, Gibbens B, Doty NS, Gomes-Pereira M, Huguet A, Stone MD, Margolis J, Peterson M, Markowski TW, Ingram MAC, Nan A, Forster C, Low WC, Schoser B, Somia NV, Clark HB, Schmechel S, Bitterman PB, Gourdon G, Swanson MS, Moseley M, Ranum PW. Non ATG-initiated translation directed by 5' microsatellite expansions. *PNAS* 2011; **108:** 260-265