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Reducing muscleblind protein levels causes decreased *DMPK* transcripts and a muscle-specific chloride channel in myotonic dystrophy type one cell line

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Abstract

From a molecular perspective, myotonic dystrophy (DM) is characterised by the presence of abnormally long units of CUG and CCUG repeats that interact with some specific nuclear proteins to form foci in patient's cells. It leads to entrapment of repeats in the nuclei to form foci in DM cells due to its association with muscleblind proteins. MBNL proteins also direct the alternative splicing of a group of genes that are aberrantly spliced in DM. In this study, MBNL downregulation was performed using short interfering RNA morpholinos designed against muscleblind (MBNL) proteins and applied to DM1 cells, as well as the wild-type and DM1 cells with a transgene splicing construct from a previous study, to assess the effect of its reduction on splicing, as well as the formation of foci in DM1 cells. The results indicate that MBNL downregulation had no effect on transgene splicing construct in wild-type cells as it showed a fluorescence ratio of 0.289 for both double downregulated MBNL and scrambled knockdown, while the untreated had a ratio of 0.228. In DM1 cells, the fluorescence ratio for the splicing construct was 0.174 for MBNL downregulation, 0.250 for scrambled and 0.251 for untreated cells. Additionally, MBNL downregulation affected foci reduction to 59.3±5.7% compared to 99.3±5.7% for scrambled. Furthermore, MBNL downregulation induced a decrease in mutant *DMPK* transcript, which was 18.0%, while scrambled and untreated were 38.4 and 53.3%, respectively. Conclusively, results from this study indicate that MBNL proteins, along with mutant *DMPK* transcript, could serve as a molecular target for therapy in myotonic dystrophy.

Introduction

Myotonic dystrophy (DM) is a neuromuscular disease which are of that has two types (DM1 and DM2), both of which have a similar molecular basis and are defined by the presence of repeat expansion units (Machuca-Tzili et al., 2005). Even though both forms of myotonic dystrophy have different clinical features, they are dominantly inherited (Machuca-Tzili et al., 2005).

For DM1, it is defined by the presence of triplet nucleotide repeat expansions (CTG) in the range of 50-3000 copies in the 3' untranslated region of dystrophin myotonia protein kinase (*DMPK*) gene, in contrast to unaffected individuals, which have 5-30 repeat copies (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). On the other hand, myotonic dystrophy type 2 has been suggested to be due to a quadruplet nucleotide repeat expansion mutation (CCTG) occurring in the intron one of the Cellular Nucleic Acid-Binding Protein (*CNBP*) gene, which is present in thousands of copies in affected persons (Liquori et al., 2001). Genetic mutations influence the length of repeats in DM and they also have effect on the response of individuals to infections (Udosen and Nya, 2017; Udosen, 2019).

Molecularly, DM is characterised by the presence of transcribed CUG and CCUG repeats which, aggregate with certain nuclear proteins to form foci in the nuclei of patient cells (Davis et al., 1997; Taneja et al., 1995). It is the lack of transportation of the transcribed mutant expanded repeat from nuclei to cytoplasm that leads to entrapment of the repeats in the nuclei, which sequester nuclear proteins to form foci in DM cells.

The major protein that interacts with CUG and CCUG repeats to form foci in cells is the

muscleblind-like (MBNL) proteins, while CUG binding protein (CUGBP) is not sequestered but is only activated in DM1 (Mankodi et al., 2001; Fardaei et al., 2001; Fardaei et al., 2002; Kuyumcu-Martinez et al., 2007). MBNL protein, in association with CUGBP proteins, regulates the alternative splicing of a group of genes which are abnormally spliced in DM (Philips et al., 1998; Mankodi et al., 2002; Savkur et al., 2001; Kimura et al., 2005; Lin et al., 2006), leading to the formation of isoforms that contribute to the disease phenotype.

A proof of study concept has demonstrated the role of transcribed expanded repeats in the induction of DM (Miller et al., 2000). This is currently being applied in many translational studies for the discovery of therapeutic agents. Additionally, reduction of MBNL proteins has also been implicated as being responsible for the DM phenotype (Lin et al., 2006; Ho et al., 2004) while its overexpression reversed some of the phenotypes associated with DM (Dansithong et al., 2005; Kanadia et al., 2006).

Reduction of MBNL protein and overexpression of CUGBP have been shown to lead to DM phenotype in mouse models (Lin et al., 2006; Ho et al., 2004; Ho et al., 2005).

The study employs a cell-based splicing assay model to investigate whether reducing MBNL proteins in unaffected cell lines can induce a DM-like phenotype by using small interfering RNAs (siRNAs) targeted against *MBNL1* and *MBNL2* genes.

Additionally, the study also assessed the effect of MBNL knockdown on the formation of foci in DM1 cells as well as determined its ability to facilitate the movement of the mutant *DMPK* abnormally long repeat transcript from the nucleus to the cytoplasm.

Material and methods

Cell culture

The human fibroblast cells utilised in this study were human DM1 patient fibroblast line (designated as KBTeloMyoD) and wild type unaffected individual fibroblast cell line (designated as NIRATelo), which had been previously telomerised and were obtained from Professor David Brook's laboratory in University of Nottingham, United Kingdom (Udosen et al., 2023). Sub-culturing of cells in flasks were carried out at 37°C incubation by utilizing 2.0ml of 0.25% Trypsin-EDTA and for 5-10 minutes under 5% CO₂. The protocols were carried out in a laminar workflow safety cabinet operating under sterilized environmental conditions (Udosen et al, 2023). The cell lines utilised for this study were performed in line with University of Nottingham research guidelines and ethics.

Ribonucleic acid fluorescence in situ hybridisation (RNA-FISH)

Cells were assessed and screened for the presence of foci in nuclei by the RNA-FISH technique, which was based on the nuclear foci concept. The foci assay was conceptualised on the unique characteristic symptoms of the mutated transcript containing abnormally long CUG repeats, which associated with protein splicing factor MBNL1, causing foci to be induced in DM cells by using a Cy3 fluorescently-tagged (CAG)₁₀ probe. RNA-FISH screening was applied to both the healthy unaffected and DM1 cells to find transcripts having abnormally long repeat units. Cells were initially rinsed with Phosphate Buffered Saline (PBS), which was in turn followed by subsequent fixation in 4% Paraformaldehyde for 15-minute

duration. The cells were subjected to three washes using PBS, followed by permeabilisation in 80% ethanol. The cells were subjected to denaturation for a duration of 10 minutes by a denaturation solution comprising of about 40% formamide along with 20x SSC in sterilised distilled water and were subsequently washed in PBS. The cells were probed with Cy3-tagged probe [(CAG)₁₀] in hybridisation solution comprising of 10% dextran sulphate, 0.2% Bovine Serum Albumin, 40% formamide, 2mM of Vanadyl Adenosine Complex, 20X SSC and approximately 1mg/ml of salmon sperm DNA. The oligonucleotide, Cy3-tagged probe, was utilised at a concentration of approximately 0.07ng/μl. The treated cells were subsequently kept in overnight incubation at 37°C and was subsequently washed three times using 5 mM MgCl₂ dissolved in PBS. It was subsequently stained for five minutes with Hoechst dye before storage at 4°C for scans and analysis on the plate reader. The plate reader utilized in this study was the Micro widefield high content imaging plate reader obtained from Molecular Devices which was fitted with Nikon objective lens 40x ELWD was employed for imaging analysis in high throughput screening of cells in RNA-FISH imaging determination of foci. The high content imaging plate reader is made up of a 488nm Argon laser, which excited GFP within the bandwidth range of 505-530nm emission filter, and 543nm Helium-Neon laser having with a long path 560nm emission filter for TRITC. The high content images obtained from the plate reader were subjected to analysis by MetaXpress software.

Cell splicing construct assay

The gene splicing constructs utilised for this study as an assay had previously been generated from genetic splicing construct comprising two reporter

genes- *Dioscorea* red fluorescent protein (DsRed) and green fluorescent protein (GFP). The second intron of *CLCN1* with its flanking exons was inserted into the pEGFP-C1 plasmid of 4.7kb size to generate pGR-*CLCN1* as reported from an earlier study (Udosen et al., 2023). For cells expressing the gene splicing construct along with its double reporters, the Ultra widefield high content image reader of Molecular Devices fitted with Nikon lens 40x ELWD was employed for imaging analysis of cellular fluorescence of the expressed transgene. GFP was viewed using 488nm laser at 525/50 emission filter; 405nm laser was used to excite DAPI through the 447/60nm filter; whereas DsRed was observed with 561nm laser which excited Texas Red using 593/40 emission filters.

Dystrophia myotonica protein kinase (DMPK) transcript analysis

The separated fractions of nuclear and cytoplasmic RNA were extracted from cultured cells by slight modification of previous protocols utilised by Hamshere et al. (1997). The cells were initially washed with freeze-cold PBS, which in turn, was followed by adding 1ml of 0.65% Nonidet P-40 (NP-40) that was formerly prepared by adding 10% (v/v) NP-40 to 1.5mM MgCl₂, 0.15M NaCl and 0.01M Tris.HCL has a pH of 8.0 in DEPC H₂O. NP-40 was added to the cells in the flask for about 1-2 minutes in order to disrupt cell membrane to release its cytoplasmic contents while its nuclear membrane was kept intact thereby giving it a grainy appearance under the microscope. The contents of the flask were isolated into a new tube as cytoplasmic extracts. Furthermore, the nuclei was dislodged by the addition of 2mls of NP-40, followed by the removal of the contents of the flask into a tube and the nuclear extracts were subsequently centrifuged to pellet it. The

supernatant was removed, and 400µl of DEPC H₂O was used to resuspend the nuclear pellet, which was followed by the inclusion of 100µl of 0.5M Tris-HCl, 0.05M EDTA and 2.5% (v/v) SDS to the nuclear extracts. For the isolation and purification of cytoplasmic extracts, 250µl of the mixture was added to it. Both fractions of nuclear and cytoplasmic RNA isolates were purified by using two equivalent volumes of phenol/chloroform extraction mixtures. The RNA isolate was immediately precipitated by centrifugation in equivalent volumes of isopropanol consisting of 0.3M sodium acetate at a pH of 5.3. The RNA pellets were rinsed using 75% ethanol, subsequently centrifuged for separation, and the supernatants were removed. Air-drying of the RNA pellet was carried out for 15-20 minutes at room temperature and subsequently dissolved in 40µl of DEPC H₂O, before its estimation and measurement by nanodrop. RNA samples obtained from DM1 fibroblasts were treated with DNaseI before cDNA synthesis by reverse transcription involving New England Biolabs guidelines prior to PCR for *Bpm1* assay. For *Bpm1* assay of *DMPK* transcript, 1/20th of cDNA was utilized for PCR involving N11 (5'CACTGTCGGACATTCGGGAAGGTGC3') the forward oligonucleotide primer while 133 (5'GCTTGCACGTGTGGCTCAAGCAGCTG3') was the reverse oligonucleotide primer. Amplification was executed by marginal modification of the protocols utilised in Hamshere et al. (1997). The conditions consisted of denaturation of DNA at the temperature of 95°C for a duration of 5 minutes, which was followed by repeated cycles of 95°C for duration of 30 seconds; annealing of oligonucleotide primers for 1 minute duration at 58°C; with extension at 72°C for a duration of 1 minute. It was subsequently followed by the final extension at 72°C for 5 minutes duration. The amplicon product was then

subjected to 98°C for a period of 1 minute, which was followed by quick cooling at 4°C for 10 minutes so as to facilitate formation of homoduplexes overnight followed by *BpmI* digestion. The final products of the restriction enzyme were separated on 3% agarose gels.

Immunoblotting

Protein isolation was obtained from the centrifuged pelleted fibroblasts using about 100-500µl of frozen RIPA lysis buffer comprising of 50mMTris-HCl at pH 8.0; 150mMNaCl; 1% NP-40; 0.5% DOC and 0.1%SDS. It was centrifuged to obtain the cell lysate contained in the supernatant, which was the crude protein, which was then removed into a fresh tube for storage. Protein was quantified using the BioRad Dc assay before western blotting. An immunoblot of protein extract samples was performed using the NuPage system obtained from Invitrogen in line with its procedures. The buffers and gels utilised were all obtained from Invitrogen. The NuPage gel system was run at 200V for 50 minutes. The blots were viewed under autoradiography machine.

Muscleblind downregulation

For MBNL downregulation, the morpholinos used to effect inhibition of its expression were:

siRNA-MBNL1 (5'CACUGGAAGUAUGUAGAGAdTdT3'), siRNA-MBNL2a (5'CACCGUAACCGUUUGUAUGdTdT3'), siRNA-MBNL2a (5'GAGGAACAUGCUCACGCUCdTdT3') and nonspecific siRNA which served as scrambled (5'GCGCGCUUUGUAGGAUUCGdTdT3').

Results and Discussion

In accordance with this proposition of this study, DM1 clones with splicing constructs showed a reduction in DsRed/GFP ratio that gave 0.174 for *MBNL1* and 2 combination knockdown, 0.250 for scrambled (nonspecific for any gene) knockdown whereas the untreated was observed to be 0.251. The result indicated that MBNL proteins reduction altered *CLCN1* splicing by inducing intron2 retention in DM1 clonal fibroblasts, thereby altering its splicing features to resemble those found in DM (Figure 1).

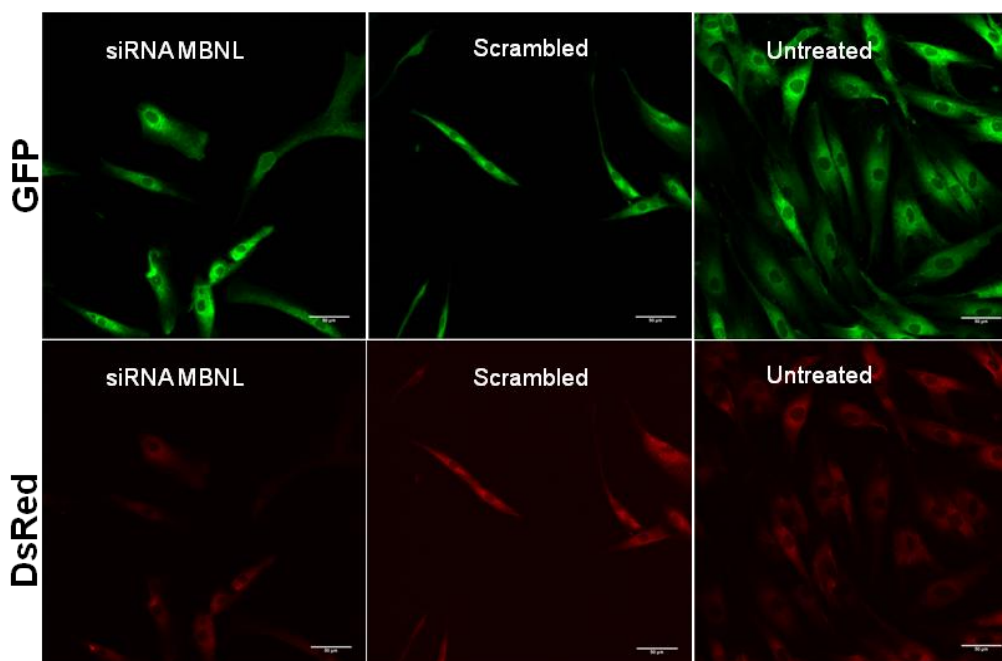


Figure 1. MBNL combination knockdown alters *CLCN1* splicing construct in DM1 cells by decreasing the intensity of DsRed fluorescence in high content imaging, in contrast to scrambled, resulting in different GFP/DsRed ratios.

On the other hand, in wild-type clonal fibroblast, MBNL combination knockdown did not exhibit any influence on DsRed/GFP ratios because both scrambled knockdown and MBNL had a similar ratio of 0.289 whereas that of untreated was 0.228 (Figure 2). The fluorescence ratios above seem to indicate that MBNL downregulation had no influence on *CLCN1* splicing regulation in wild-type clonal cells. To verify the possibility that MBNL1 and 2 proteins were not actually expressed in the wild type as well as in DM1 fibroblast clones, immunoblotting of protein extracts from cells was performed on samples for *MBNL1* and 2 double knockdown, along with that of scrambled knockdown. Immunoblotting results

standardised to tubulin indicated that MBNL1 was about 90% decreased in wild-type clones and greater than 95% reduction was achieved in DM1 fibroblast clones.

Therefore, MBNL 1 and 2 downregulation alters *CLCN1* splicing construct in DM1 fibroblasts but not in wild type fibroblasts. DM1 fibroblasts were made to undergo *MBNL* knockdown to test its influence on nuclear foci number, which was an assay used in a previous study for therapeutic screening (Ketley et al., 2013).

The objective was to determine if MBNL protein reduction would result in a decrease in nuclear foci, as was demonstrated in a previous study by Dansithong et al. (2005).

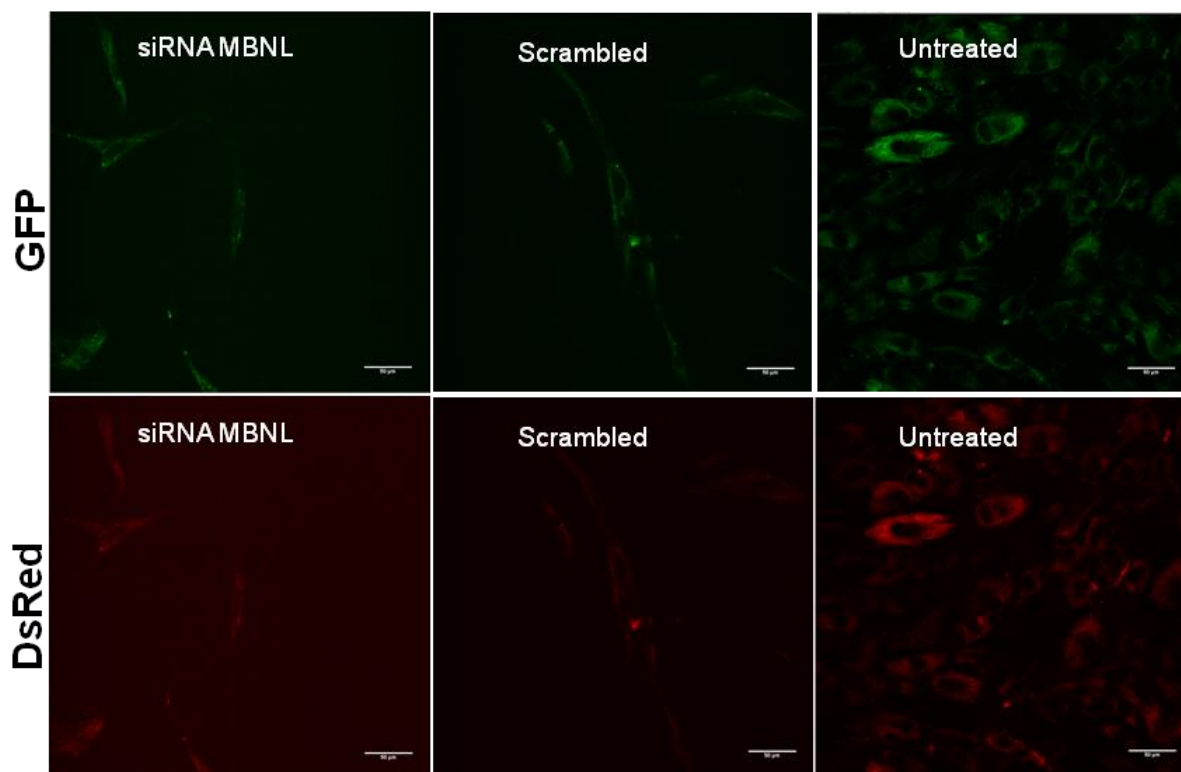


Figure 2. MBNL protein reduction in clonal cells of wild type expressing *CLCN1* gene splicing construct, indicating no change in GFP/DsRed fluorescence ratio in contrast to scrambled and untreated cells utilized as controls.

In line with this proposition, combination knockdown of *MBNL1* and *MBNL2* exhibited $59.3\pm 9.7\%$ decrease in the foci of DM1 fibroblasts, in contrast with scrambled ($99.3\pm 5.7\%$), which served as a negative control (Figure 3). The result indicated that *MBNL1* and *2* downregulation could lead to a reduction of foci in the nuclei of DM cells. Since *MBNL1* and *MBNL2* combination knockdown was demonstrated to elicit $59.3\pm 9.7\%$ decrease in nuclear foci number, the concept was also applied in *Bpm1* polymorphism restriction assay so as to assess if the MBNL protein reduction effected the translocation of mutant *DMPK* having abnormally long repeats from nucleus into cytoplasm of DM1 fibroblasts.

siRNA combination knockdown of *MBNL*, which was carried out alongside the nuclear foci assay, indicated that the mutated form of the *DMPK* transcript did not undergo transportation to the cytoplasm of DM1 cells (Figure 4). Nevertheless, the *MBNL* combination knockdown induced a decrease in the proportional amount of the mutated *DMPK* transcript. Analysis by Genescan screen of the normal/wild type transcripts as well as the mutant *DMPK* transcripts indicated that the proportional percentages of the mutated allele occurring in the nucleus to be were 18.0, 38.4 and 53.3% for the *MBNL* combination knockdown, scrambled downregulation and untreated control, respectively.

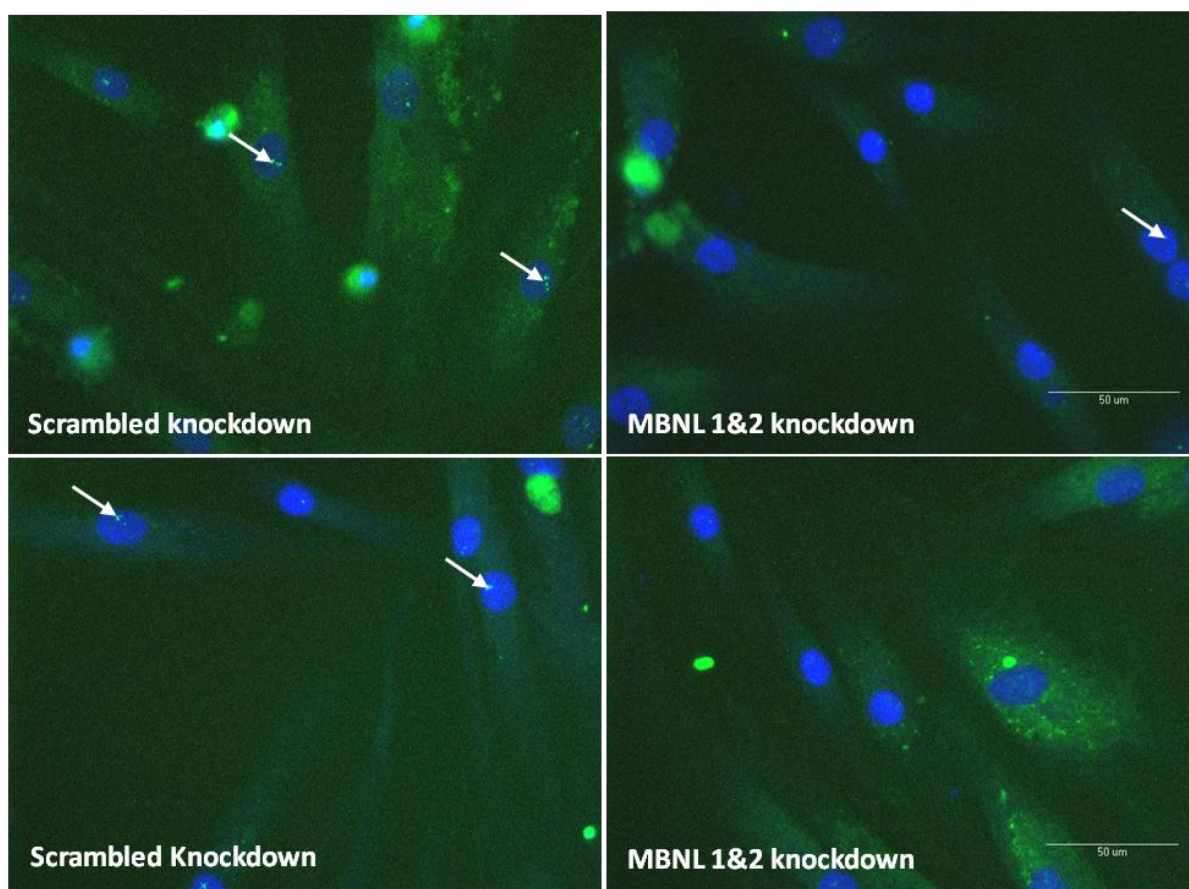


Figure 3. *MBNL1* and *2* combination knockdown decreases foci number in the nucleus, whereas scrambled knockdown had no effect on foci number in DM1 cells. The arrows point to nuclear foci in the cells.

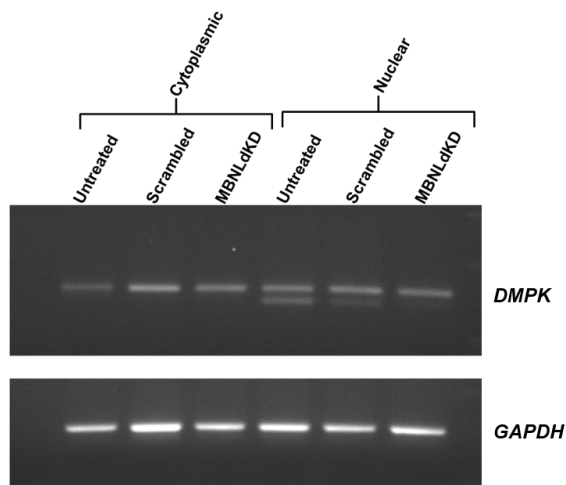


Figure 4. *MBNL1* and *2* downregulation did not translocate mutated *DMPK* transcripts into the cytoplasm but resulted in their decrease, in contrast with controls, which are the scrambled knockdown and untreated

Based on previous studies which demonstrated that *MBNL1* downregulation in wild type cells alter *IR*, *cTNT*, and *SERCA1* splicing (Ho et al., 2004; Dansithong et al., 2005; Hino et al., 2007), *MBNL1* and *2* double knockdown was performed in both *CLCN1* clonal cells of the wild type and DM1 fibroblasts to determine its influence on *CLCN1* splicing in these clonal lines. The aim was to determine if *MBNL* knockdown in these clones would alter the fluorescence ratios (DsRed/GFP) in these cells. The splicing concept had been used in a previous work to test the efficacy of compound treatment on a subset of genes abnormally spliced in the myotonic dystrophy type 2 cell line (Udosen et al., 2023; Udosen, 2020;). From the results obtained from this study, combination knockdown of *MBNL1* and *MBNL2* did not affect the splicing of the *CLCN1* gene construct in wild-type cells, as reported in other studies (Ho et al., 2004; Dansithong et al., 2005; Hino et al., 2007). This could be due to the fact that *MBNL 1* and *2* downregulation alone does not account for the induction of myotonic dystrophy

symptoms. It is possible that *MBNL* protein acts in association with *CUGBP1* proteins to cause myotonic dystrophy, hence the inability of *MBNL* downregulated wild-type clonal cells to exhibit DM-like features.

MBNL1 and *MBNL2* proteins have been previously shown to colocalise with foci in nuclei of DM cells (Mankodi et al., 2001; Fardaei et al., 2001; Fardaei et al., 2002). In line with this proposal, *MBNL1* and *MBNL2* protein downregulation resulted in a decrease in the number of foci in DM1 cells. In addition, the combined *MBNL1* and *MBNL2* protein downregulation led to a decrease in the proportional representation of the mutated *DMPK* transcript in the nucleus even though it did not affect its translocation to the cytoplasm. From this study, the effect of muscleblind protein downregulation on *DMPK* transcript has indicated that it could serve as a promising molecular target for therapeutic intervention.

However, limitation of the study is in relation to *DMPK* transcript which is only applicable to myotonic dystrophy type one (DM1) cells and not in myotonic dystrophy type 2 (DM2) cells. This is due to the fact that DM2 condition is induced by mutation in *CNBP* which is different from that of DM1.

Conclusion

Taken together, this study indicate that *MBNL1* and *MBNL2* proteins is one of the prominent factors accountable for induction of myotonic dystrophy-like symptoms and could act in association with other nuclear proteins to induce molecular features associated with the disease. As a consequence, muscleblind proteins, along with the mutant *DMPK* transcript, could serve as a molecular target for therapeutic intervention.

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Authors' contributions

I. Udosen: Conceptualization; Investigation; Writing –original draft.

M. Akpanabiatu: Resources; Writing – review & editing.

I. Sandy: Software; Writing – review & editing.

Conflict of interest

The authors hereby declare that there is no conflicting interest involved in this manuscript.

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