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A Cell-Based Double Reporter Gene Splicing Assay for Therapeutic Screening in Myotonic Dystrophy

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Abstract

The study has developed a model splicing construct assay system based on splicing misregulation, one of the major molecular features associated with myotonic dystrophy. The splicing construct assay has double reporters for intron 2 splicing in chloride channel (*CLCN1*). The *CLCN1* transgene splicing construct assay was used to transfect wild type and DM fibroblast cell lines and the clones generated showed that it enabled quantification of splicing efficiency in transgene construct. Validation of the DM fibroblasts containing transgene splicing construct was performed by differentiating the DM fibroblasts into myoblasts which exhibited a switch in *CLCN1* splicing construct which was consistent with that associated with myotonic dystrophy (DM) condition. The myoblast derived from fibroblasts cell-based gene-splicing assay was subsequently applied in therapeutic screening in small throughput screens of 113 compounds which identified Protein Kinase C inhibitors- hypericin and Ro-31-8220 as potential therapeutic agents. The *CLCN1* gene-splicing assay is a good model system for application in therapeutic screening in myotonic dystrophy because its double reporters facilitated quantification of effect putative drug on correction of misregulated splicing.

Keywords: myotonic dystrophy, splicing assay, chloride channel, therapeutic screening, transgene constructs.

Introduction

Myotonic dystrophy, classified as type 1 (DM1) or type 2 (DM2), is a neuromuscular disease molecularly defined by mutations in RNA induced by expanded repeat units (1,2). The other major molecular feature is misregulation of alternative splicing (3). Muscleblind-like (MBNL)1 protein depletion leads to misregulation of splicing in many genes and in some cases it resulted in myotonic dystrophy (DM) phenotypes (3). MBNL1 sequestration has been implicated in misregulated splicing of chloride channel 1 gene (*CLCN1*), cardiac troponin T (*cTNT*) and insulin receptor (*IR*) leading to myotonia, cardiac conduction defects and insulin resistance respectively observed in myotonic dystrophy (DM) patients (4, 5,6,7). MBNL1 had affinity for binding to CUG repeats in (8) while MBNL2 and 3 were found to colocalize with RNA foci in DM1 and DM2 cells (8,9,10). CUG-BP (CUG repeat RNA Binding Proteins) has been demonstrated to be phosphorylated by protein kinase C (PKC) in DM1 to form the active isomers- CUG-BP1 and CUG-BP2 (11,12) which are involved in regulation of alternative splicing. CUG-BP1 protein levels are not elevated in wild type skeletal muscles but they are increased in DM1 muscle cultures (12). This explains the interaction of CUG-BP and MBNL proteins in the regulation of cardiac troponin T (13) and insulin receptor pre-mRNAs (12). The alteration in the relative ratios of these protein factors has been suggested to lead to defect in splicing patterns in DM (12,13,14).

Since toxic RNAs acquire dominant negative effect through sequestration of muscleblind-like (MBNL) proteins mediate the disease condition, assay development for therapeutic target include: (1) disruption of interactions in foci between MBNL and RNA CUG repeats in DM1 or CCUG repeats in DM2; (2) destruction or removal of mutant RNA containing repeat expansions; (3) correction of misregulated splicing; (4) increased dosage of MBNL and (5) inhibition of CUGBP1.

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Many assays for myotonic dystrophy have been developed. One assay uses RNA repeat units-MBNL1 interactions (15). The study utilized resin-bound dynamic combinatorial chemistry (RBDCC) in which a fluorescently labelled RNA repeat having the sequence Cy3-CCG-(CUG)10-CGG was monitored for interactions with a compound library. The RBDCC is a competitive assay that utilizes the affinity of resin-bound compounds to bind the fluorescently labelled RNA by competing with each other. However, it was not possible to determine if hit compounds obtained from this assay could reverse spliceopathy associated with myotonic dystrophy (DM) nor whether the hits obtained in this assay are toxic when applied to DM patients' cell cultures.

Gel-shift assays have been utilized in identifying compounds that inhibit CUG expanded repeats interaction with MBNL1 (16,17). In a study by Wheeler and Colleagues (2009), a CAG25 antisense oligonucleotide was demonstrated to bind CUG repeat hairpins by blocking CUG repeat-MBNL1 complexes to form a stable CUG repeat-morpholino heteroduplex in a gel-shift assay (17). Subsequently, Warf and Colleagues (2009) used a similar technique to identify pentamidine as lead compound that disrupted MBNL1-CUG repeat interactions in a gel-shift assay (16). However, gel-shift assays may not be sufficient in screening therapy of DM as toxicity and side effects of compounds cannot be determined hence any compounds identified in this way may need further testing in cell cultures to assess their effects on cell viability as well as in rescuing of splicing defects and foci clearance in DM patients' cells.

Another screening assay for identification of compounds that bind to CUG repeats exploited isothermal titration calorimetry (ITC) to identify compounds that could bind to T-T and U-U mismatches in CTG or CUG repeats by forming hydrogen bonds (18). This assay was also shown to disrupt complex structure formation between MBNL1 and poly(CUG). It is not possible to determine if assays of this nature can identify compounds with no toxic or side effects unless applied to DM cells.

A modular design of ligands that bind to RNA repeat with high affinity and specificity has been reported in a technique called Huisgen dipolar cycloaddition reaction (HDCR) (19,20,21,22). HDCR was used to direct ligands on microarray surfaces and its interaction with RNA repeats units were analysed bioniformatically. Even though, molecules identified by this approach were found to be permeable in mouse muscle cells C2C12, the issue is whether its cellular uptake and localisation, especially with respect to repeat RNA in nucleus would be effective in DM cells.

Thus far only one assay based on intron2 splicing of *CLCN1* in DM cells has been reported for its application in DM-related High Throughput Screening (HTS) which identified AKT V inhibitor triciribine as a putative therapy (23). However, this assay used in HTS was limited in its ability to determine the level of compound effect on correcting intron2 splicing in a quantitative manner. In this study, we have developed a model splicing construct assay system with double reporters for intron splicing in *CLCN1*. The *CLCN1* splicing construct assay is a double reporter system allowing quantification of the splicing efficiency of transgene construct in both DM1 fibroblast and DM1 myoblast.

Materials and Methods

Cell lines used in the study include mouse muscle cells (C2C12), a wild type human cell line and a DM1 fibroblast cell line containing inducible MyoD which can be differentiated into myoblasts in the presence of doxycyclin. All human fibroblasts used in this project had been previously telomerised. Culture passage of cells in flasks were performed using 2.0ml of 0.25% Trypsin-EDTA incubated at 37 °C and 5% CO_2 for 5-10 minutes. These procedures were performed in a Walker laminar flow safety cabinet under aseptic conditions.

The splicing constructs used in this study for the assay were previously generated from gene splicing construct containing reporters- green fluorescent protein (GFP) and Dioscorea red fluorescent protein (DsRed). Intron 2 of *CLCN1* with its flanking exons as were cloned into pEGFP-C1 plasmid of 4.7kb size to generate pGR-CLCN1 (Figure 1).

Transgene splicing constructs were confirmed by sequencing. Subsequently, gene transfer experiments were carried out using transgene constructs to transform *E. coli* DH5α cells which were grown as overnight cultures and its plasmid DNA were harvested using QIAGEN plasmid DNA purification kit protocol prior to transfection studies in C2C12 and human fibroblasts.

The C2C12 cell lines and human fibroblasts were transfected with plasmid DNA using the Amaxa Nucleofection kit in accordance with manufacturer's instructions.

Clones of human fibroblasts and C2C12 lines were selected after the cells were transfected with transgene constructs. Selection of clones was carried out in G418 (neomycin) under 200ug/ml for fibroblast cells.

DM1 fibroblasts used in this study had been previously infected with Lentivirus containing a MyoD inducible vector and in the presence of doxycyclin were induced to differentiate into myoblasts by addition of doxycyclin to a concentration of 2ug/ml for 6-8 days

DNA extraction from fibroblasts cell lines was carried out using a Puregene-Gentra DNA purification kit in line with protocols specified by the manufacturer. DNA samples were quantified either on a nanodrop spectrophotometer or on agarose gel using a quantitative *λHind*111 ladder

The PCR analysis was performed in a final volume of 20µl and consisted of 1.0µl of DNA (50ng/µl), 2.0µl of 10x PCR buffer [made up of 750mM Tris-HCl pH 8.0, 200mM $(NH_4)_2SO_4$, 0.1%(v/v0 Tween 20] , 1.2µl of 15mM $MgCl_2$, 0.8 µl of 20mM dNTPs, 1.0µl each of 10pmol forward and reverse primers, 13.7 µl of distilled water and 0.3 µl of 5U/ µl of ABgene red hot Taq polymerase. The primers used to amplify *CLCN1* in pGR-CLCN1 were T1F (5'AAAGACCCCAACGAGAAGC3') and T1R (5'GATCCTCATCCTTGCTGTCC3') while the primers-*MIR*1-2f (5'TGCCATGCTTCAGGTAAAC3') and *MIR*1-2r

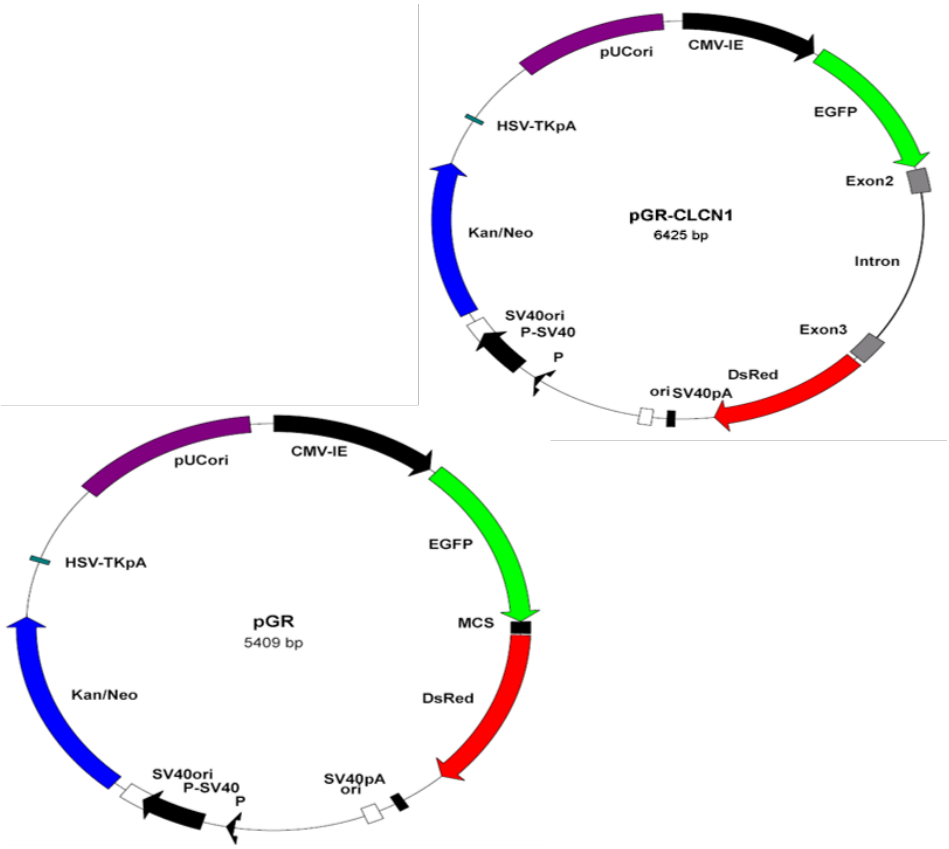


Figure1. Transgene constructs pGR-CLCN1 on pEGFP-C1 plasmid used for transformation of *E. coli* DH5α and subsequent transfection in fibroblasts for the splicing assay. pGR serves as the positive control of the splicing assay.

(5'CACGTAGAAAGAAGCAAGAGC3') were included to amplify microRNA1-2, a house keeping gene. The PCR products were resolved on 2% agarose gel.

For *CLCN1* PCR, the following conditions were used: Denaturation at 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute; 55°C annealing of primers for 1 minute and extension at 72°C for 2 minutes. Final extension was carried out at 72°C for 10 minutes.

The PGR-vector PCR involved the following conditions: Denaturation at 95°C for 5 minutes followed by 30 cycles (genomic DNA) of 95°C for 1 minute; 59°C annealing for 1 minute and 72°C extension for 2 minutes with final extension carried out at 72°C for 10 minutes.

Cells were treated with compounds in both 96-well plates. For small throughput screens, initial concentrations of 48μM, 8 μM and 1.6 μM were used. The optimal concentrations of putative hit compounds- D8 (hypericin) and D9 (Ro-31-8220) used in the screens were 50 μM and 5μM respectively. The compound library screen consisted of 113 small molecules made up of phosphatases and kinases. The kinase inhibitor collection consisted of 80 compounds including inhibitors of BTK, CaM Kinase II, CDK, CKI & II, EGFR, GSK, IKK, IR, JAK, JNK, MAPK, MEK, MLCK, PI 3-kinase, PDGFR, PKA, PKC, RAF, SAPK, Src-family and VEGFR. The phosphatase inhibitor library was composed of 33 compounds including PP2B designated as calcineurin, CD45, CDC25, JSP-1, PP1, PP2A, PRL-1, PRL-3, PTEN etc.

The imaging of cells were performed using both Inverted and confocal microscope. Live cell imaging was performed using Zeiss inverted fluorescent microscope fitted with confocal scans together with Olympus CK2 confocal microscope with 4x, 10x and 40x objectives.

Green and red fluorescence in fixed cells was examined using Zeiss axioscop2 mot plus fluorescence microscope fitted with a digital camera having DAPI, brightfield, phase contrast as well as green and red fluorescent filters. Cells were viewed using 10x objectives. Analysis of fluorescent images was carried out using Openlab software.

The fluorescent microscopes used to view fixed cells include Zeiss LSM510 confocal on a Zeiss Axiovert 100M microscope, Zeiss plan neofluar 40x/1.3 oil objective and Zeiss plan Apochromat 63x/1.4 oil objective. Images from these microscopes were analysed using LSM software.

For high content imaging of cellular fluorescence in small throughput screens of cells with transgene splicing double reporter construct, Molecular Devices Ultra widefield high content plate reader fitted with Nikon 40x ELWD was used for high content imaging of cellular fluorescence. The 488nm laser was

used to view GFP at 525/50 emission filter; DAPI was excited with 405nm laser using 447/60nm filter; while DsRed was observed using Texas Red which was excited by 561nm laser with 593/40 emission filters.

Results

These splicing construct assays were designed to screen for compounds that could correct the splicing misregulations that take place in myotonic dystrophy. Transgenes constructs were based on alternative splicing events involving introns reported in muscle-specific chloride channel (*CLCN1*).

The intron 2 of *CLCN1* contains a stop codon whose splicing results in translation of the single open reading frame of the transgene constructs to give chimeric product with green and red fluorescent activities. However, if no splicing occurs, the intron 2 is retained and only green fluorescent activity is produced as a result of stoppage of translation by a stop codon in pGR-CLCN1 (figures 2). To test the proof of splicing concept above, the splicing constructs were tested in mouse muscle cells, C2C12 in a transient transfection study.

The application of the splicing concept showed cells fluorescing after transfection of both constructs which demonstrated splicing of the introns from the relevant gene constructs to give green and red fluorescent activities (Figure 3). This finding is consistent with the splicing model shown in figure 2 below.

After validation of the splicing constructs in C2C12 cells, transgenes were subsequently transfected into wild type fibroblasts as well as DM1 fibroblast lines. The transfected cells were then selected in 200µg/ml G418 and many of the clones were scanned for fluorescence. DNA from the clones was tested by PCR amplification to confirm the presence of the relevant transgenes using primers T1F and T1R for *CLCN1*, and *MIR*1-2f and *MIR*1-2r for microRNA1-2, a house keeping gene, in genomic DNA to generate products of 208bp and 312bp respectively, in a duplex reaction (figure 4).

A total of 44 clones were obtained from human fibroblasts, pGR-CLCN1 was successfully introduced into wild type and DM cell lines. Twenty-six pGR-CLCN1 positive clones were derived from wild type fibroblasts and 5 clones from DM fibroblasts. The pGR-CLCN1 positive clones were subsequently optimized for therapeutic screening in this study.

The wild type and the DM1 fibroblasts clones showed splic-

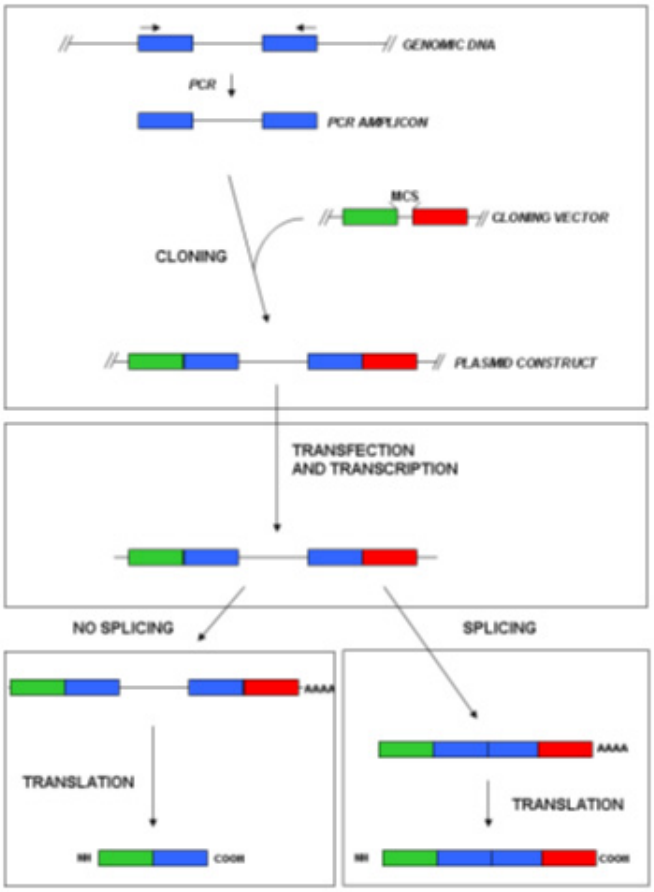


Figure 2. Schematic model of the splicing concept for transgene construct when splicing occurs leading to intron exclusion (bottom right) to produce both GFP and DsRed or no splicing (bottom left) resulting in intron inclusion to produce only GFP.

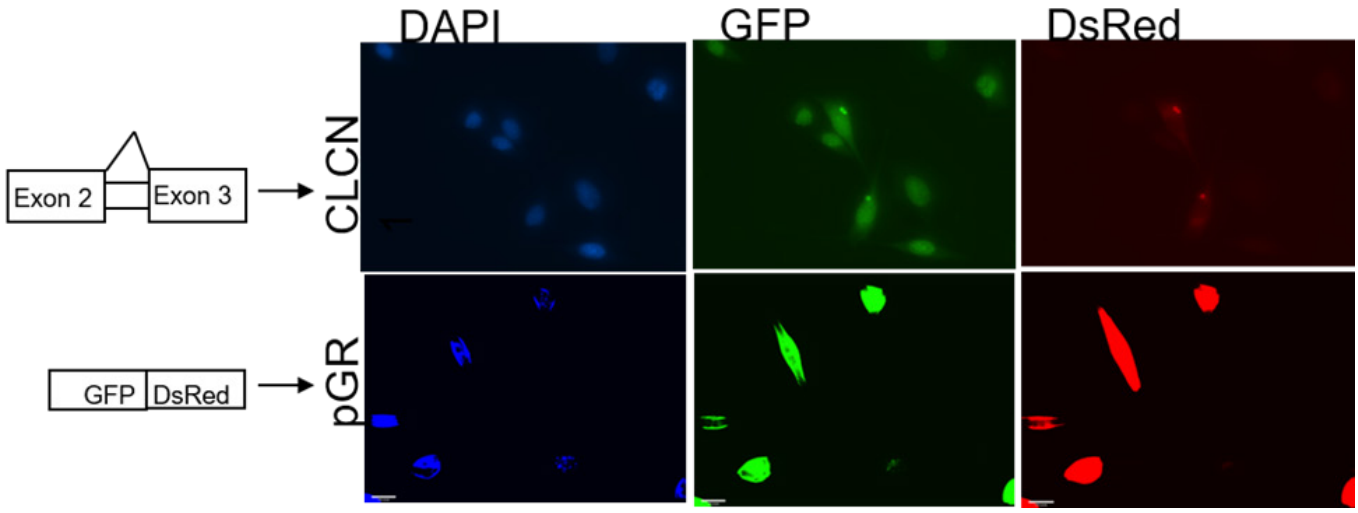


Figure 3. Mouse muscle C2C12 cells transfected with splicing constructs express both green and red fluorescence. This indicates the transgene construct has been spliced. pGR is the positive control for the study.

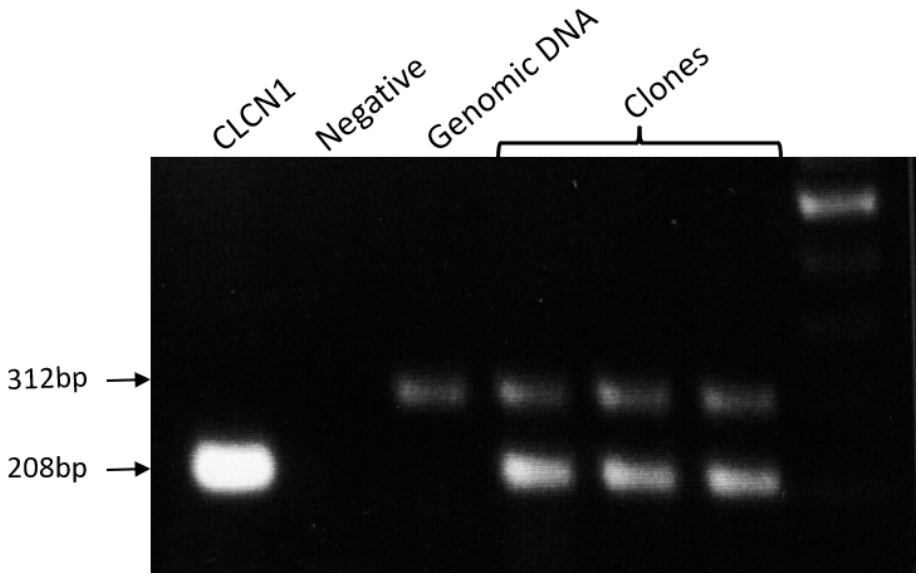


Figure 4. PCR amplification of cell clone DNA resolved on an agarose gel to confirm the presence of splicing construct pGR-CLCN1 in representative agarose gel for wild type and DM fibroblasts.

ing of the pGR-CLCN1 transgene constructs in fluorescent scans that was similar to that demonstrated in C2C12 showing the splicing of introns to produce the predicted fluorescence (figure 5).

The clones obtained from these lines were validated before use in therapeutic screening. Validation involved differentiating DM1 clones containing splicing constructs to myoblasts to determine whether there are changes in the splicing ratio of green: red fluorescence. The fluorescence ratio (DsRed fluorescence/green fluorescence) was used as a measure of the rate of splicing in the *CLCN1* splicing constructs.

The clone of DM1 fibroblasts containing a *CLCN1* splicing construct was subjected to differentiation by the presence of doxycyclin which induces MyoD expression, a muscle differentiation factor, to convert the fibroblasts into myoblasts. The DM1 cell line used in this study had leaky expression of MyoD hence they can only differentiate into myoblasts and not myotubes because only 90% of the cells were infected with inducible MyoD.

Based on the splicing concept, it was hypothesized that the fluorescence ratio in the DM1 clone containing the *CLCN1* splicing construct, which gives an indication of the extent of

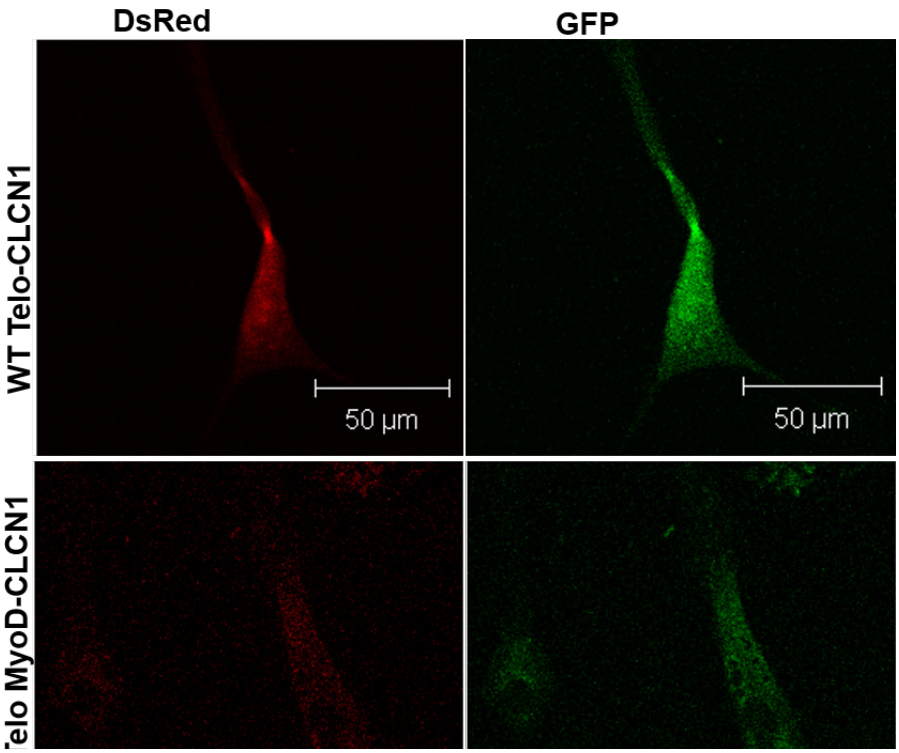


Figure 5. Fluorescent images of the fibroblast cell clones transfected with transgene construct pGR-CLCN1 showing splicing of the construct and resulting fluorescence.

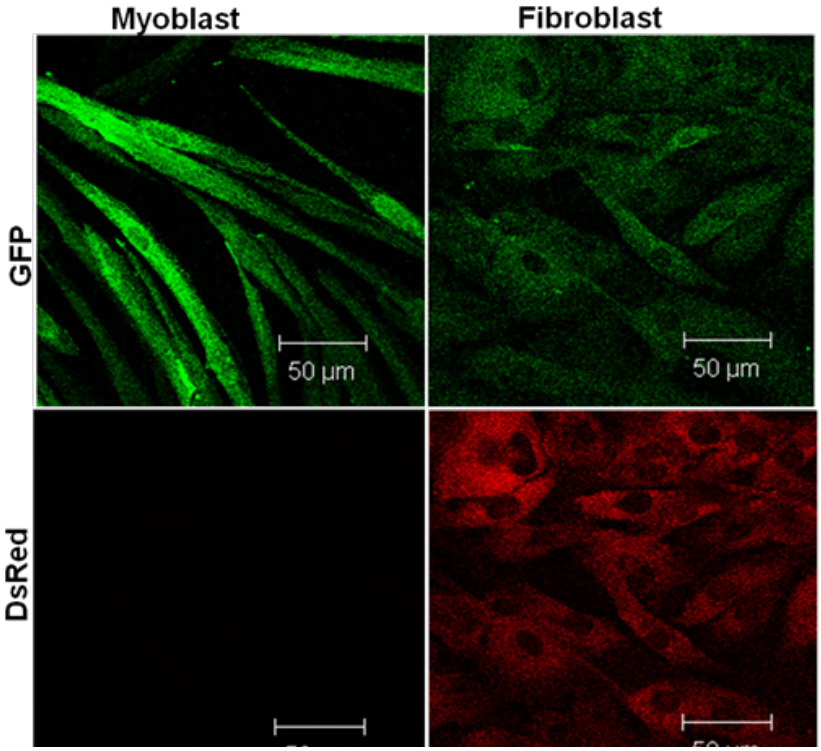


Figure 6. Intron2 of *CLCN1* splicing transgene is spliced out in DM1 fibroblasts as shown by the expression of both GFP and DsRed in these cells. When fibroblasts undergo differentiation into myoblasts, intron2 of the splicing transgene is retained resulting in expression of only GFP. This represents a developmental switch from normal splice patterns in DM1 fibroblasts to DM1 splicing pattern in myoblasts.

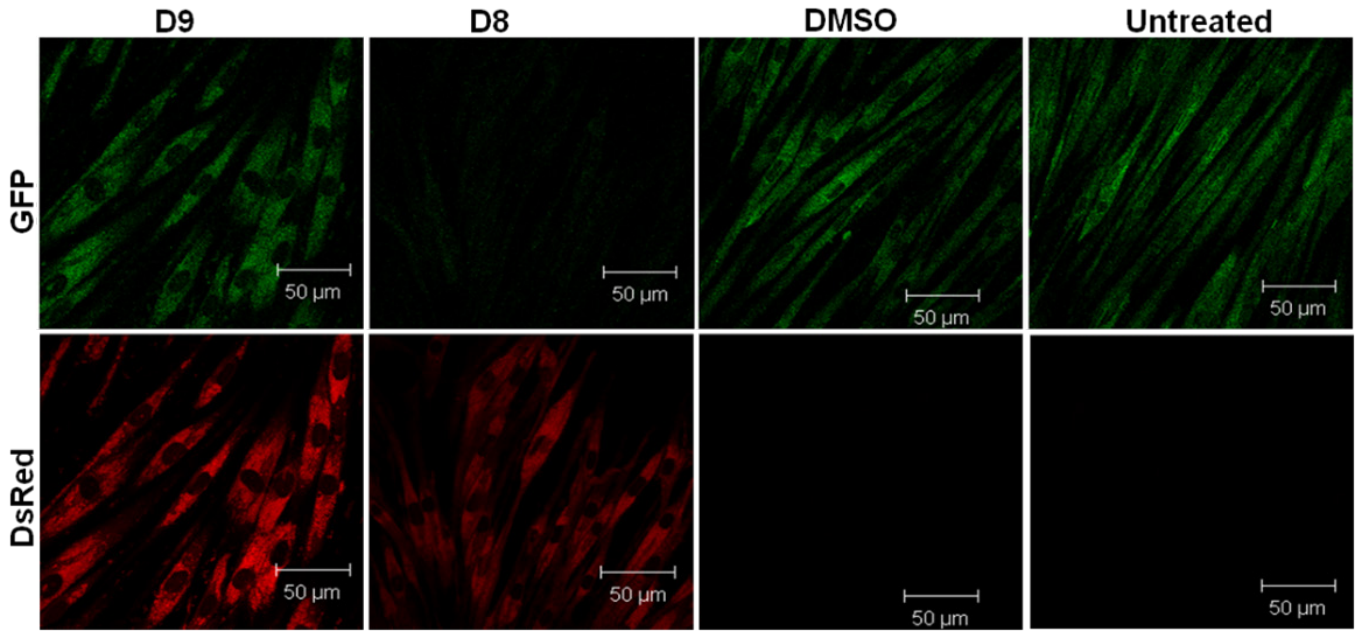


Figure 7. Hypericin (D8) and Ro-31-8220 (D9) treatment reverses DM-like splicing patterns of intron2 retention of the *CLCN1* splicing transgene in DM1 myoblasts. Confocal images are shown in treated and mock control dimethylsulphoxide (DMSO) and untreated myoblasts.

splicing, would be altered as is reported in studies of splicing of transcripts upon differentiation of DM1 cells (12,23).

Consistent with this proposal, the fluorescence ratio (ie %DsRed to GFP) observed in the DM1 cells containing the *CLCN1* splicing construct changed from 1.0 (100.0%) in fibroblasts to 0.0 (0.0%) in confocal scans showing no red fluorescence in these cells after differentiation into myoblasts (figure 6).

The switch in splicing by the expression of green fluorescent protein (GFP) and red fluorescent protein (DsRed) in DM1 fibroblasts compared to the GFP-only expression in myoblasts, derived from the same fibroblast clonal lineage, is due to the retention of intron2 of the *CLCN1* splicing construct in myoblasts. This leads to the termination of transgene transcript translation resulting in GFP expression only in the DM1 myoblasts. The intron2 retention in the DM1 myoblasts mimics the splicing pattern associated with DM. This demonstrates that the clonal fibroblasts, which undergo developmental change to a DM-like splicing pattern on differentiation, is suitable for use in compound screening for DM therapy.

Based on a previous study using nuclear foci for drug screens (24), a library screen of 113 compounds consisting of inhibitors of phosphatases and kinases were used in this study to screen for molecules that could reverse splicing defects in myotonic dystrophy (DM) exemplified by DM1 myoblasts.

The *CLCN1* splicing construct assay to assess compounds identified in the primary screening assay based nuclear foci assay. Analysis of the expression of GFP and DsRed by DM1 myoblasts on treatment with compounds served as a test for the effectiveness of compound treatment on correcting the DM-like splicing pattern.

The positive hits in Small Throughput Screens obtained from the screening library were PKC inhibitors designated in the compound library as hypericin (D8) and Ro-31-8220 (D9). Both hypericin and Ro-31-8220 treatments of differentiating DM1 fibroblasts (DM1 myoblasts) restored *CLCN1* misregulated splicing in DM1 myoblasts (figure 7).

The confocal images showed the presence of both green and red fluorescent cells in contrasts to mock control (DMSO treated) and untreated cells which exhibit only green fluorescence. The expression of only GFP in controls is an indicator of *CLCN1* misregulated splicing due to the retention of intron 2 which contains a premature termination codon leading to the truncated translation of the *CLCN1* open reading frame.

The assay was optimised on the Molecular Devices Ultra scanning device used for small throughput screening. The ratios of DsRed/GFP were quantified and analysed using metaXpress software multiwavelength cell scoring. Hypericin showed a greater DsRed/GFP ratio of 1.14 which was three times greater than that of Ro-31-8220 which was 0.36. Both dimethylsulphoxide (DMSO) treatments and untreated had fluorescence intensity ratios of DsRed/GFP of 0.06 and 0.07, respectively, which were significantly less than the measures following hypericin and Ro-31-8220 treatments. The small throughput

screening scans confirmed the result from confocal scans that both hypericin and Ro-31-8220 treatments improved intron2 splicing in differentiated DM1 reporter construct clones. Furthermore, hypericin showed a greater effect on splicing than Ro-31-8220 with the *CLCN1* reporter splicing assays in small throughput screening as demonstrated by higher DsRed/GFP ratio.

Discussion

Spliceopathy (splicing misregulation) is a major molecular feature in myotonic dystrophy hence it is a primary target for assay development for therapeutic reversal of the disease condition. Spliceopathy of *CLCN1* and *SERCA1* have been used in screening therapy studies of DM (16,25,26).

Therapeutic reversal of spliceopathy in DM has been demonstrated using antisense against CUG repeats (27), antisense oligonucleotides (17,25), siRNA against *MBNL* (5), MBNL1 overexpression (28,29) and morpholino antisense oligonucleotide (26). This justifies the use of reversal of spliceopathy in DM as a measure of identifying molecules or drugs that can alleviate DM phenotypes.

The model splicing assay system developed in this project is the splicing construct assay with double reporters for intron splicing. The *CLCN1* splicing construct assay used for compound screens in this study has the advantage over those of O'Leary and Colleagues (23) because it is a double reporter system allowing quantification of the splicing efficiency of transgene construct in both DM1 fibroblast and DM1 myoblast. The quantification of splicing is absent from the luciferase *CLCN1* construct of O'Leary and Colleagues therefore the degree of efficacy of compound or drug can be estimated in the system reported here. Furthermore, the bichromatic fluorescent reporter system developed in this study differs from those used by Orengo and Colleagues (30) by having both reporters in the same open reading frame as opposed to having the reporters in alternate reading frames used in their study. The splicing construct assay model system has been optimised for small throughput screening and the ratios of DsRed/GFP fluorescence intensity serve as a measure of the efficacy of compound treatment on *CLCN1* intron2 spliceopathy in this system. The splicing system developed in this study for myotonic dystrophy has been effective in identifying putative compounds that could be potential therapy for the disease. Recently, an assay based on quantification mutant *DMPK* expanded repeat RNA has been reported eventhough it is yet to be applied in HTS format (31). The assay system of this study could be optimised for use in High Throughput Screens. The splicing assay was used to identify two potential therapeutic compounds which were protein kinase C inhibitors- hypericin and Ro-31-8220. The Protein Kinase C inhibitor, Ro-31-8220, had been used in a functional study to test the role of CUG-BP1 in DM in an inducible cardiac-specific mouse model of the disease (32). Administration of Protein Kinase C inhibitors to this mouse model was observed to relieve cardiac conduction defects as well as cardiac abnormalities. In addition, these inhibitors were found to reduce misregulated splicing events in exon21 of *Ank2* and exon16 of *Mtmr3* which are specifically regulated by CUG-BP1 (32). Furthermore, it was demonstrated that Protein Kinase C activation by expanded CUG repeat induces hyperphosphorylation of CUG-BP1 leading to an increased half-life of the protein. It is the hyperphosphorylated forms of CUG-BP1 that are involved in misregulation alternative splicing (11,12). Taken together these studies demonstrate that inhibition of Protein Kinase C activity could be a useful target for therapy of DM hence its justification for inclusion in the library of compounds used for myotonic dystrophy therapy screening in this study. The Protein Kinase C (PKC) inhibitors- Ro-31-8220 and hypericin had been shown in our previous study to remove nuclear foci, affect MBNL distribution and influence CELF-1 protein levels by PKC-independent mechanism in DM cells (24). Currently, there is ongoing work to search for small molecules for DM therapy (Timchenko, 2022). Therefore, the cell-based double reporter gene splicing assay developed and applied to small molecules screening test in this study is a good model assay system for therapeutic screening for myotonic dystrophy.

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