GSK3β is a new therapeutic target for myotonic dystrophy type 1

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Myotonic dystrophy type 1 (DM1), an incurable, neuromuscular disease, is caused by the expansion of CTG repeats within the 3′ UTR of *DMPK* on chromosome 19q. In DM1 patients, mutant *DMPK* transcripts deregulate RNA metabolism by altering CUG RNA-binding proteins. Several approaches have been proposed for DM1 therapy focused on specific degradation of the mutant CUG repeats or on correction of RNA-binding proteins, affected by CUG repeats. One such protein is CUG RNA-binding protein (CUGBP1). The ability of CUGBP1 to increase or inhibit translation depends on phosphorylation at Ser302, which is mediated by cyclin D3-CDK4. The mutant CUG repeats increase the levels of CUGBP1 protein and inhibit Ser302 phosphorylation, leading to the accumulation of CUGBP1 isoforms that repress translation (i.e., CUGBP1[REP]). Elevation of CUGBP1[REP] in DM1 is caused by increased GSK3β kinase, which reduces the cyclin D3-CDK4 pathway and subsequent phosphorylation of CUGBP1 at Ser302. In this review, we discuss our recent discovery showing that correction of GSK3β activity in the DM1 mouse model (i.e., *HSA^LR* mice) reduces DM1 muscle pathology. These findings demonstrate that GSK3β is a novel therapeutic target for treating DM1.

Introduction

Myotonic dystrophy type 1 (DM1) is an autosomal dominant inherited disease that affects many tissues. In skeletal muscle, this condition leads to weakness, atrophy, and myotonia.[1] In addition to skeletal muscle symptoms, patients with DM1 develop arrythmias and cardiomyopathy in the heart, neurological abnormalities, and insulin resistance. DM1 is caused by a mutation in the *dystrophia myotonica* protein kinase *(DMPK)* gene, which results in an expansion of CTG trinucleotide repeats within its 3′ UTR.[2,3] CTG expansions are highly unstable and vary in length in normal individuals from 5 to 35 repeats. In patients with DM1, the length of CTG expansions, which correlates with the severity of clinical symptoms, varies from 50 to several thousand repeats.

Development of DM1 therapy requires elucidation of the molecular mechanisms by which CTG repeats lead to DM1 pathology. Studies have established that CTG repeats lead to DM1 pathology by causing the accumulation of mutant *DMPK* mRNA transcripts containing an expanded array of CUG repeats that target RNA-binding proteins, thereby changing their activities and altering RNA homeostasis in DM1 cells.[4-8] The most studied RNA-binding proteins affected by CUG repeats are CELF1 (also known as CUGBP1) and MBNL1. CUGBP1 is a member of the family of CUGBP and ELAV-like factors, whereas MBNL1 belongs to the muscleblind-like (MBNL) family of proteins.[9-11]

The mutant CUG repeats primarily cause two toxic events in DM1 cells. First, the aggregation of mutant *DMPK* transcripts, possibly due to disruption of their subsequent processing,[11,12] can lead to sequestration of MBNL1 proteins, thereby reducing their splicing activity[11,13] (**Fig. 1**). Second, the mutant CUG repeats
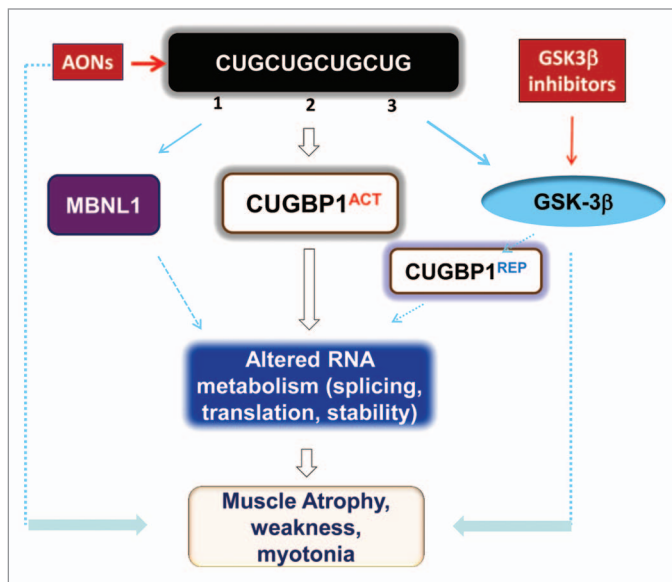
Figure 1. Toxic events caused by mutant CUG repeats in DM1 cells and possible therapeutic approaches for their correction. Mutant CUG repeats cause three toxic molecular events: (1) sequestration of MBNL1, (2) higher levels of CUGBP1, leading to elevation of the active form of CUGBP1 (CUGBP1^ACT), and (3) elevation of active GSK3β, which reduces cyclin D3 and converts a portion of CUGBP1^ACT to CUGBP1^REP. Thus, both CUGBP1 forms are elevated in DM1. Reduced MBNL1 and increased CUGBP1^ACT lead to deregulation of mRNA splicing, translation, and stability. Futhermore, increased CUGBP1^REP may reduce mRNA translation in stress granules. Taken together, these molecular changes lead to myotonia, weakness, and muscle atrophy. Administration of GSK3β inhibitors reduces DM1 muscle histopathology, weakness, and myotonia similar to degradation of mutant CUG repeats by AONs.

increase CUGBP1 stability, leading to elevated levels of this protein in DM1 cells[14,15] (**Fig. 1**). This stabilization is mediated by an increase in PKC-mediated phosphorylation of CUGBP1 and its subsequent binding to soluble CUG repeats.[14,16]

However, these mutant CUG repeats also disrupt CUGBP1 phosphorylation by other kinases. CUGBP1 contains a number of serine and threonine residues that regulate its activity and intracellular localization. Ser28 is normally phosphorylated by Akt to control the nuclear-cytoplasmic distribution of CUGBP1.[17] Another important phosphorylation site is Ser302, which is located within the spacer between RNA-binding domains 2 and 3. Ser302 phosphorylation, which is mediated by cyclin D3-dependent kinase 4 (CDK4),[17] promotes CUGBP1 binding to eukaryotic translation initiation factor 2α (eIF2α) and the subsequent association of CUGBP1 with polysomes.[17-19] Because of its positive role in activating the translation of several mRNAs, encoding

p21, HDAC1, MEF2A, and cyclin D1, phospho-Ser302 CUGBP1 was designated as CUGBP1^ACT. Although CUGBP1^ACT binds to eIF2α, it does not affect global translation.

We found that a significant proportion of CUGBP1 in DM1 cells is un-phosphorylated at Ser302.[17] In contrast to the phosphorylated form, un-phosphorylated Ser302 CUGBP1 binds to inactive (phospho-Ser51) eIF2α, thereby forming the inactive CUGBP1^REP-eIF2α complex. This repressor complex is localized within stress granules (SGs) to trap mRNAs and delay their translation.[17,20] We found that one CUGBP1 target, *MRG15*, which encodes a protein, involved in DNA repair and chromatin remodeling, is bound by CUGBP1^REP in DM1 cells, leading to reduced MRG15 protein levels.[17] These data showed that CUGBP1^REP may repress the translation of many additional mRNAs in cytoplasmic aggregates or SGs, and that Ser302 phosphorylation of CUGBP1 by cyclin D3-CDK4 may act to switch the function of CUGBP1

from repressor to activator of translation. Interestingly, both forms of CUGBP1 are elevated in DM1. In addition to regulating translation, CUGBP1 controls mRNA splicing and stability.[17-19,21-24] Therefore, elevation of CUGBP1^ACT in DM1 may increase the translation of some mRNAs and change the splicing and stability of CUGBP1 targets.

Our recently published work showed that the increase in CUGBP1^REP in DM1 cells is due to elevated levels of activated GSK3β.[25] Consistent with this, GSK3β levels are also increased in the skeletal muscle of *HSA^LR* mice, which express tracks of 250 CUG repeats within the 3′ UTR of human skeletal actin and is commonly used model of CUG repeat RNA mediated pathology in DM1.[5,25] GSK3β has many substrates, including cyclin D3.[26] Phosphorylation of cyclin D3 by GSK3β in DM1 patients and *HSA^LR* mice results in reduced cyclin D3 protein levels because phosphorylated cyclin D3 is targeted for degradation. In agreement with this reduction in cyclin D3 in DM1 cells, CUG repeats convert CUGBP1^ACT to CUGBP1^REP.

Once alterations to the GSK3β pathway were discovered, we investigated whether GSK3 inhibitors can correct the pathology of DM1. Analysis of *HSA^LR* mice treated with inhibitors of GSK3, namely lithium and TDZD-8, showed that elevated GSK3β plays a crucial role in DM1 pathology. We found that both inhibitors significantly increased grip strength in *HSA^LR* mice and improved DM1 muscle histology.[25] Moreover, treatment of *HSA^LR* mice with lithium or TDZD-8 significantly reduced variability in myofiber size (**Fig. 2A-C**), as well as the number of central nuclei in *HSA^LR* muscle[25] (**Fig. 2D**). Based on these data, we concluded that the elevated GSK3β is the third major toxic event in DM1 pathogenesis (**Fig. 1**).

Initially, we expected that deregulation of the GSK3β-cyclin D3-CDK4-CUGBP1 pathway may be responsible for muscle atrophy in DM1. However, treatment with lithium and TDZD-8 also reduced myotonia in *HSA^LR* mice.[25] Thus, it is possible that the correction of CUGBP1 activity may not only "normalize" the translational activity of
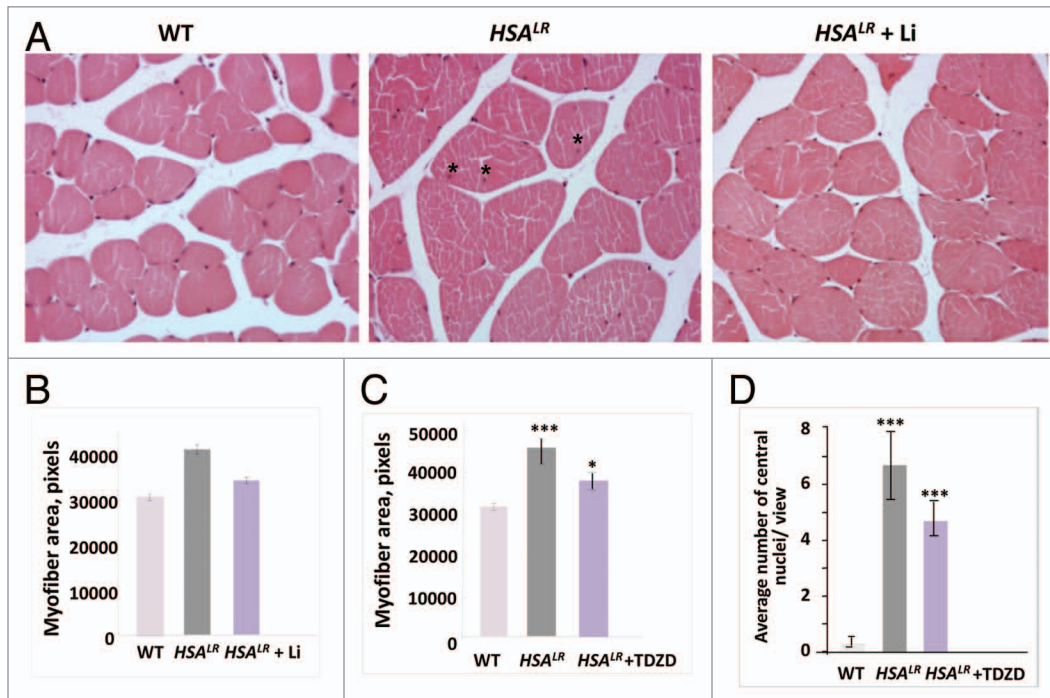
Figure 2. Correction of muscle histopathology in *HSA*[LR] mice treated with GSK3 inhibitors, namely lithium and TDZD-8. (**A**) Hematoxylin and eosin staining of muscle sections (*gastrocnemius*) from age-matched 6-mo-old WT, untreated *HSA*[LR] mice, and lithium-treated *HSA*[LR] mice. Internal nuclei in the muscle of untreated *HSA*[LR] mice are indicated by asterisks. (**B**) Lithium reduces myofiber size variability in *HSA*[LR] mice. Myofiber area was compared in *gastrocnemius* from age-matched 6-mo-old WT mice and *HSA*[LR] mice untreated and treated for 2 weeks with lithium. The y-axis shows the average myofiber area in pixels. $p < 3.36 \times 10^{-16}$ (untreated *HSA*[LR] mice vs. WT mice); $p < 1.10 \times 10^{-7}$ (treated vs. untreated *HSA*[LR] mice). (**C**) TDZD-8 treatment reduces myofiber size variability in *HSA*[LR] muscle. Myofiber area was increased in 4-mo-old *HSA*[LR] mice relative to WT mice (***$p < 5.88 \times 10^{-6}$). The myofiber area was reduced in *HSA*[LR] mice after TDZD-8 treatment. *$p < 0.02677$ (treated *HSA*[LR] mice vs. untreated). (**D**) Treatment of *HSA*[LR] mice with TDZD-8 reduces the number of central nuclei in the skeletal muscle (*gastrocnemius*) of *HSA*[LR] mice. The number of central nuclei was counted in six randomly selected 20 × views and the average values are shown. The average number of central nuclei per view was increased in *HSA*[LR] mice (***$p < 4.616 \times 10^{-8}$) relative to WT mice. However, treatment with TDZD-8 reduced the number of central nuclei. ***$p < 0.000387$ (treated *HSA*[LR] mice vs. untreated).

CUGBP1, but also its splicing activity. This correction may lead to the correction of the splicing of one myotonia-associated target of CUGBP1, the chloride channel.[23] Although we found that lithium converts CUGBP1[REP] into CUGBP1[ACT] in *HSA*[LR] muscle, further investigation is necessary to determine whether the GSK3β-cyclin D3-CDK4 pathway plays a major role in reducing myotonia in DM1 mice or whether GSK3β inhibition affects other pathways in these mice. Taken together, we found that GSK3 inhibitors reduce DM1 pathology in *HSA*[LR] mice similar to the degradation of mutant CUG repeats by antisense oligonucleotides (AONs)[27-29] (**Fig. 1**).

Our initial experiments in which 6-mo-old *HSA*[LR] mice were treated with GSK3 inhibitors were based on protocols using short treatment durations, specifically 2–7 d for TDZD-8 or 2 weeks for lithium.[25] We noticed that grip strength increased and that myotonia was reduced in the treated mice immediately after treatment. However, once treatment was completed, *HSA*[LR] mice developed muscle weakness again. These results suggest that, to maintain "normalized" grip strength in adult *HSA*[LR] mice, GSK3 inhibitors must be administered continuously if the treatment protocols tested in our recent study are used.[25] In contrast, myotonia in these mice remained lower for several weeks after cessation of treatment.[25] The mechanisms responsible for the long beneficial effect of the GSK3 inhibitors on myotonia in *HSA*[LR] mice remain to be studied. Interestingly, the effects of these inhibitors on grip weakness were more pronounced in 6-week-old *HSA*[LR] mice. Treatment of 6-week-old *HSA*[LR] mice with TDZD-8 for 1 week significantly increased the grip strength in these mice when evaluated at 3 mo of age.[25] These data suggest that inhibition of GSK3β in *HSA*[LR] mice at a pre-symptomatic age may be beneficial to reducing the incidence of DM1 pathology in adult *HSA*[LR] mice.

Conclusions

In summary, our study found increased levels of GSK3β in the skeletal muscle of DM1 patients and *HSA*[LR] mice.[25] This alteration seems to be a key event in DM1 pathology because GSK3 inhibitors can reduce dysfunctional muscle pathology in the DM1 mouse model. It remains to be determined whether GSK3β plays a role in the development of other DM1 symptoms, such as insulin resistance and defects in cardiac and neurological functions. Studies have demonstrated that GSK3β is increased in other conditions, including type 2 diabetes mellitus.[30] Therefore, elevated GSK3β may contribute to the development of

insulin resistance in DM1. Since GSK3 inhibitors are used to treat neurological disorders such as bipolar disease,[30] these compounds may also improve neurological dysfunction in patients with DM1 in addition to correcting skeletal muscle defects.

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