Current targeted therapeutic strategies for oculopharyngeal muscular dystrophy: from pharmacological to RNA replacement and gene editing therapies

Aida Abu-Baker¹, Nawwaf Kharma², Christian Neri³, Sarah Rasheed¹, Patrick A. Dion¹,⁴, Luc Varin², and Guy A. Rouleau¹,⁴

1Montreal Neurological Institute and Hospital, McGill University, Montréal, Québec H3A2B4, Canada
2Biology Department, Concordia University, 7141 rue Sherbrooke O., Montreal, Québec, H4B 1R6, Canada
3INSERM, Laboratory of Neuronal Cell Biology and Pathology, Center for Psychiatry and Neuroscience UMR 894 and University of Paris Descartes, Equipe d'accueil 4059, 75014 Paris, France
4Department of Neurology and Neurosurgery, McGill University, Montréal Québec, Canada

Correspondence: Guy A. Rouleau
Montreal Neurological Institute and Hospital, 1033 Pine avenue, Montréal, Québec H3A2B4, Canada
Email: guy.rouleau@mcgill.ca

Abstract

Oculopharyngeal muscular dystrophy (OPMD) is a midlife onset hereditary disease affecting skeletal muscles. It is characterized by progressive eyelid drooping, swallowing difficulties and proximal limb weakness. The distinct pathological hallmark of OPMD is the presence of filamentous intranuclear inclusions (INI) in patient’s skeletal muscle cells. OPMD is caused by a mutation in the poly (A) binding protein nuclear 1 protein (PABPN1) gene, located on chromosome 14q. The normal PABPN1 gene has a (GCG)6 repeat encoding a polyalanine stretch at the 5’ end, while in OPMD patients this repeat is expanded to (GCG)8-13. Currently there is no effective treatment for OPMD. In this review, we discuss our current treatment strategies for OPMD. We present three experimental therapeutic approaches: pharmacological, RNA replacement, and gene editing. Our scientific findings could ultimately lead to an effective therapy for OPMD patients.

Keywords: Oculopharyngeal muscular dystrophy, Pharmacological treatment, RNA replacement therapy, Gene editing, Poly-alanine disorders, Protein aggregation, PABPN1
Oculopharyngeal muscular dystrophy (OPMD) is a mid-life onset (~45 years) primarily autosomal dominant muscular disease with minor neuronal involvement [1]. It is characterized by progressive swallowing difficulties, eyelid drooping and serious proximal limb weakness at later stages. The pathological hallmark of the disease is the presence of intranuclear inclusions (INI) in the muscle biopsies of patients [2, 3]. The highest prevalence of OPMD is in the French-Canadian population, where 1/1,000 people are at risk, though the disease is found worldwide. In 1990, our group began collecting samples from affected families and, in 1998, we identified the poly(A) binding protein nuclear 1 protein (PABPN1) gene as causative [4, 5]. OPMD results from short expansions of a 5’ trinucleotide alanine coding repeat located at the N-terminus end of the protein. The normal PABPN1 protein has 10 alanines and the mutated (exp)PABPN1 has 11-18 [4].

The PABPN1 protein has 306 amino acids. In addition to the polyalanine stretch, PABPN1 contains a proline-rich region in the N-terminus, an RNA-binding domain in the central region and an arginine-rich C-terminus. PABPN1’s well-recognized role in mRNA polyadenylation and transport is attributed to its ability to bind poly(A) tails with high affinity [6, 7]. A number of recent studies have revealed additional roles for the protein, including transcription regulation and poly(A) RNA export [8, 9]. PABPN1 appears to control gene expression in various tissues, including muscles [10, 11]. The depletion of PABPN1 in mouse primary muscle cells leads to myogenic defects and reduced differentiation [11]. PABPN1 expression in human cells has also been reported to be autoregulated through a mechanism of controlled intron retention coupled to pre-mRNA decay [12]. Moreover, PABPN1 was shown to suppress alternative cleavage, polyadenylation sites [13] and the regulation of long non-coding RNAs (lncRNA) [8].

Since our publication of the first PABPN1 mutations in 1998, a striking array of cellular and molecular mechanisms has been proposed to contribute to the pathogenesis of OPMD (Figure 1). Proposed mechanisms include sequestration of cellular factors by the mutant protein [14, 15], defects in the potential clearance pathway (i.e. chaperones, and ubiquitin-proteasome pathway UPP) [14, 15], alterations in transcription, histone acetylation alteration [16], aging-associated factors [17], Wnt pathway perturbation [18], and aberrant protein-protein interactions [19]. The toxic gain-of-function mechanism of OPMD supports the hypothesis that disease onset and progression is dependent upon the expansion of the polyalanine tract. The mutant protein becomes misfolded due to the presence of expanded tract as it allows a transition toward a distinct β-sheet conformation that may cause toxicity in several ways. It may exert toxicity as a monomer or it may self-associate to form toxic oligomers. The oligomers can assemble into larger aggregated species and ultimately they are deposited in macromolecular intracellular inclusions. The context of the polyalanine tract within the PABPN1 protein has an essential role in the disease process. The composition of regions flanking the repeats can alter the biochemical and biophysical properties of expPABPN1. Differences in the production and/or clearance of expPABPN1 in eyelid and pharyngeal muscles might be determinants of age-related muscle vulnerability in OPMD. These might be used as therapeutic targets. For example, as polyalanine expansions lead to misfolded PABPN1 protein, structural refolding assisted by enhanced chaperone activity might be beneficial. An increased degradation of expPABPN1 and aggregates via proteasomes could reduce the amounts of toxic species inside the cell.

Despite our understanding of the normal functions of PABPN1 protein, and the proposed mechanisms contributing to OPMD pathogenesis, the mechanisms whereby mutated expPABPN1 is toxic to muscle is still a mystery. Alanine is a highly hydrophobic amino acid, often found in the cores of proteins [20], and polyalanine oligomers are extremely resistant to chemical denaturation and enzymatic degradation [21]. Our initial hypothesis was that expanded polyalanine tract of expPABPN1 forms β-pleated sheets that give rise to toxic INI. As our work progresses, we are discovering that soluble expPABPN1 may be the most critical species for toxicity [22].

OPMD shares many common features with other polyalanine diseases, polyglutamine diseases, muscle dystrophic diseases, and diseases caused by RNA binding proteins (Figure 2). In addition to OPMD, at least eight other inherited diseases are now caused by genes with polyalanine expansions [23]. All the affected genes in polyalanine diseases, except PABPN1, code for transcription factors that play important roles in early development [23]. OPMD was the first polyalanine disorder identified that leads to INI in muscle nuclei of patients[2]. Later studies demonstrated the presence of protein aggregates in several polyalanine diseases [24-26]. Hydrophobic polyalanine tracts in the normal range are considered to be flexible spacers...
Aspects of OPMD are similar to the common features of 4 families of disorders: polyalaine expansion disorders; polyglutamine disorders; muscular dystrophies; and RNA-binding protein disorders.

OPMD is a trinucleotide repeat disorder sharing common features with polyglutamine disorders. Both OPMD and polyglutamine diseases are coding triplet trinucleotide diseases and both are late onset of disease. Both alanine and glutamine are hydrophobic amino acids which are able to form β-sheet structures, and induce intracellular protein aggregation. The most striking difference between OPMD and polyglutamine diseases is the repeat lengths. Short expansions (>2) of the polyalanine stretch in wild-type PABPN1 cause OPMD while much longer expansions of polyglutamine (>35) are required to cause neurodegenerative diseases [27]. Another difference is that in OPMD the pathogenic effects are mostly observed in skeletal muscle cells as opposed to neuronal tissues, in the case of polyglutamine disorders.

OPMD is part of a family of muscular dystrophies. Muscular dystrophy diseases have been associated with mutations in nuclear proteins (e.g. PABPN1), structural proteins, signaling molecules and enzymes as well as mutations that result in aberrant processing of mRNA or alterations in the post-translational modifications of proteins. Muscular dystrophies are a heterogeneous group of inherited disorders that share similar clinical features and dystrophic changes in muscle biopsies [28]. Key features of dystrophic muscle include central nuclei, small regenerating fibres and accumulation of connective tissue and fatty tissue. There are nine types of muscular dystrophy, each involving an eventual loss of strength, increasing disability, and possible deformity: Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), Congenital, Distal, Emery-Dreifuss, Facioscapulohumeral (FSHD), Limb-Girdle, Myotonic, and OPMD [29]. The diverse mechanisms of these diseases all lead to the common outcome of muscle degeneration and weakness, and therapy aimed at stimulating muscle growth may be effective against these common pathways.

RNA-binding proteins (RBPs) are essential players in RNA metabolism. They are involved in key cellular processes from pre-mRNA splicing to mRNA translation. There are more than 800 RBPs encoded by the human genome, composed of approximately 40 different types of domain motifs. Genetic studies have identified a large number of mutations in RBP genes in a wide range of human diseases, suggesting the critical importance of RBPs in human biology and pathogenesis [30]. There are a growing number of examples in which genetic mutations in RNA binding proteins lead to human diseases particularly neurological and muscular degenerative disorders. For example: CUGBP, Elav-like family (CELF) and Muscleblind-like (MBNL) in myotonic dystrophy (DM1); fused in sarcoma (FUS) and TAR DNA-binding protein (TDP-43) that have pathogenic roles in amyotrophic lateral sclerosis (ALS); fragile X mental retardation 1 gene in Fragile X syndrome (FXS); and survival motor neuron gene (SMN1) in Spinal muscular atrophy (SMA) [31]. Interestingly, most of these diseases are neurological and/or neuromuscular. Toxic RNA gain-of-function mechanisms contribute to pathogeneses such as FXS and OPMD. Conversely, loss-of-function mechanisms are associated with disorders such as DM1 and SMA. Interestingly, PABPN1 is an RNA binding protein containing two RNA binding domains. Mutations that increase the stability of interactions between an RNA species and RNA binding protein substrates can cause disease. The discovery of new disease-causing mutations in RNA binding proteins provides a range of therapeutic targets.

Despite the monogenic nature of the disease and the time that has elapsed since the identification of the mutations responsible for OPMD, as well as the progress in understanding the molecular mechanisms, there is no available treatment for patients afflicted with the disease at the moment. Therapy is limited to the management of symptoms. Various surgical approaches can transiently improve swallowing. Ptosis can also be surgically corrected. These procedures do not affect the progression of the disease and the symptoms usually reappear.
Over the last few years, several potential treatment approaches have emerged [16, 18, 32-38], some of which are described in Table 1. Given that these treatment strategies are limited to reducing the symptoms of the disease, it is vital that new therapies are developed that directly target the underlying mutation in OPMD. Therefore, recently, we have employed three parallel strategies to further develop effective therapies for OPMD. Based on our understanding of the pathogenic mechanisms of OPMD, we aimed to target the mutated protein pharmacologically using selective drugs. In addition, we have developed two gene therapy strategies based on: a) RNA biology (gene knockdown and replacement) using antisense oligonucleotides; and b) gene editing approach using the CRISPR/Cas9 system to precisely correct the mutation of OPMD (Figure 3).

Here, we highlight our major translational medicine advances in developing effective treatment for OPMD and discuss our three approaches, namely; drug therapy, RNA replacement therapy, and gene editing therapy (Figure 3). OPMD is a particularly attractive target for DNA and RNA based therapies as it is an autosomal dominant disease resulting from mutation on one allele. Thus, in concept, eliminating expression of the mutant PABPN1 allele would entirely prevent the muscular pathology that it otherwise cause. Because the mutant PABPN1 messenger RNA (mRNA) transcript would be selectively targeted (using RNA molecules and the optimized codon wild-type PABPN1), the normal transcript would remain unaffected and able to mediate the normal functions of PABPN1 that may be critical for muscle function. Emerging genome editing technologies, such as CRISPR-Cas9 systems, open a new avenue for precisely and easily editing the mutation on the DNA sequence of PABPN1 on chromosome 14. On the other hand, our pharmacological approach targets the defective protein in OPMD by using selective candidate drugs.

### Treatment Strategies

Over the last few years, our group and others have generated several of models for OPMD ranging from mammalian cell models to transgenic mouse models in order to test various hypotheses, resulting in a better understanding of the molecular basis of the disease [1, 3, 18, 36, 39, 40]. This work has provided a foundation of knowledge permitting us to develop treatment strategies that target OPMD pathological mechanisms. In order to determine which treatments may prove potentially beneficial in OPMD patients, we are currently testing and validating our three novel therapeutic strategies in different established cell and animal models as well as OPMD patients' myoblasts and lymphoblast cell lines.

#### Pharmacological approach: drug therapy

**LiCl (Wnt Pathway)**

The Wnt signaling pathway has been shown to have a strong effect on cell proliferation and survival and it may contribute to the overall process of myogenesis [41]. Wnt signaling directs cell fate determination in various tissues, including skeletal muscle [42]. β-catenin is the central signaling molecule of the canonical Wnt pathway, and when complexed with lymphoid enhancer factor/T-cell factor transcription factors in the nucleus, it activates the transcription of target genes. The regulation of β-catenin activity is thought to occur via a cytoplasmic multiprotein complex that includes the serine/threonine kinase glycogen synthase kinase-3β (GSK-3β), which phosphorylates β-catenin, marking it for degradation by the proteasome [43-45]. An important consequence of GSK-3β suppression is the stimulation of both the Wnt/beta-catenin and Akt/mTORC1 pathways [46]. Recently, GSK-3β inhibi-

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<td>Zinc sulfate</td>
<td>Essential mineral nutrient</td>
<td>Cells</td>
<td>(Wang et al., 2005) [32]</td>
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<td>8-hydroxyquinoline</td>
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<td>Cells</td>
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<td>Modified antibody protein</td>
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<td>Antibiotic (FDA)</td>
<td>Cells &amp; fruit fly</td>
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<td>Trehalose</td>
<td>Sugar</td>
<td>Cells &amp; mice (clinical trial underway)</td>
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<td>Cystamine</td>
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<tr>
<td>Lithium chloride</td>
<td>Epilepsy/bipolar disorder drug (FDA)</td>
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<td>Sirtinol</td>
<td>A selective sirtuin histone deacetylase inhibitor</td>
<td>Cells &amp; C.elegans</td>
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<td>Autologous myoblast transplantaion</td>
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Table 1. Examples of different strategies tested for potential to treat OPMD.
Towards OPMD Therapy

One of the first described inhibitors of GSK-3β is lithium [46]. Our recent study shows that lithium chloride (LiCl), an FDA approved drug already used for the treatment of epilepsy and bipolar disorder patients, can rescue the cell death induced by the expression of expPABPN1 in C2C12 cells. This protection appears to be associated with the increase of β-catenin following the inhibition of GSK-3β by the compound [18] (Figure 4A and B). Furthermore, this effect was also observed in primary cultures of mouse myoblasts expressing expPABPN1. A similar effect on β-catenin was also observed when lymphoblastoid cells lines derived from OPMD patients were treated with LiCl [18]. We believe manipulation of the Wnt/β-catenin signaling pathway may represent an effective route for the development of future therapy for patients with OPMD.

Valproic acid (Histone deacetylase inhibitors)

Cellular histone acetylation levels are a critical determinant of transcriptional activity [48]. Histone acetylation levels are determined by the relative activity of two opposing classes of proteins: histone acetyltransferases (HAT) and histone deacetylases (HDAC). Recent reports show that regulation of the histone acetylation profile, as a mechanism modulating transcription, may explain in part the toxicity observed in expanded polyglutamine-induced toxicity [49-51]. Importantly, these studies provide evidence that pharmacological restoration of histone acetylation may mitigate the toxic effects of expanded polyglutamine.

Abu-Baker et al.
Given the similarities between OPMD and polyglutamine disorders, we have begun to explore the possible role of histone acetylation in OPMD. Normal PABPN1 protein has been suggested to regulate the expression of muscle-specific genes through its interaction with the transcription co-activator, SKIP [10], which is also an adaptor protein implicated in transcriptional repression complexes containing HDAC1 [53]. The expression of several muscle-specific genes is also regulated through specific HAT and HDAC enzymes [54, 55]. Our previous findings reveal that expPABPN1 alters the profile of histone acetylation in *C. elegans* and mammalian cells [16]. Furthermore, manipulating levels of histone acetylation significantly altered the developmental delay seen in the nematode model of OPMD, as well as the muscle nuclei loss seen in mammalian cells, supporting a critical role for this system in OPMD pathogenesis [16].

Sirtuins are known to be involved in histone acetylation, though this effect is not fully characterized, and to act as sensors of the metabolic state of cells and organisms [56]. The sirtuins are also known to regulate muscle gene expression and differentiation by inhibiting MyoD and MEF2 factors [55, 57, 58].

Based on this data, we believe HDAC inhibitors, such as valproic acid (VPA) may represent new potential therapeutic avenues for OPMD. VPA is an FDA approved compound and a direct inhibitor of (HDAC) class I and II [59, 60]. Therefore, recently, we have tested VPA (Sigma) in a stable C2C12 cell model of OPMD. Using a combination of live cell imaging and biochemical measures, we observed that VPA confers its long-term protective effects on muscle cell survival, proliferation and differentiation in mammalian cells (Figure 4C). VPA testing in transgenic OPMD mouse model is currently underway and our preliminary data show that treatment results in an improved performance in exercise testing, reduced muscle fiber damage as well as a reduction in INI in skeletal muscle fibers. VPA is proving to have considerable therapeutic potential for OPMD patients.

**ADAPT-232 and USP14 Inhibitor** *(Chaperones and Ubiquitin-Proteasome Pathway UPP)*

The expansion of polyalanine may induce a misfolding of PABPN1 and increase its propensity for aggregation by conferring a toxic gain of function. Early studies of OPMD revealed large INI containing mutant PABPN1 as a common pathogenic marker [3, 21]. These aggregates recruit ubiquitin and chaperones such as HSP70 [14] as well as other nuclear proteins [19]. They are marked for ubiquitin proteasome degradation but for unknown reasons are not degraded. This suggests that the structure of the aggregates renders them resistant to the misfolded protein clearance pathway, leading to cellular dysfunction [14]. This theory could account for the susceptibility of muscles associated with OPMD, since these highly metabolically active cells may be more sensitive to perturbations in cellular machinery. Our further observation that the formation of protein aggregates does not entirely correlate with cell death [22] have contradicted the theory of inclusion body toxicity, adding to the increasing evidence suggesting that they
might instead serve a protective role within cells. It is now generally accepted that the toxic species are smaller, less visible micro-aggregates [61].

Our group was the first to report the direct involvement of the UPP pathway and molecular chaperones in OPMD pathogenesis [14]. Later studies in OPMD animal models and patients confirmed our findings and identified UPP deregulation as the predominant molecular defect in the disease [15, 62]. Based on the evidence that UPP and molecular chaperones contribute to the disease pathogenesis, we sought to explore the efficacy of two drugs known to activate this pathway: ADAPT-232 (kindly provided by Dr. Panossian, Sweden) and USP14 inhibitor (Sigma).

It has been shown that the adaptogenic activity of ADAPT-232 is associated with key mediators of stress response, e.g., heat-shock-proteins (Hsp70), involved in the regulation of homeostasis, oxidative stress, energy metabolism, cognitive function, and activation of the immune system during fatigue and exhaustion [63, 64]. ADAPT-232 (Chisan®) is a traditional natural herbal medicinal product consisting of a fixed combination of extracts from Rhodiola rosea root, Schisandra chinensis berry, and Eleutherococcus senticosus root [65]. A recent study showed that Adaptogens stimulate Hsp72 expression in neuroglial cells [66]. Here, we tested ADAPT-232 for its protective effects against the cell death associated with expPABPN1 in our established C2C12 stable transfecant model. We found that treating cells expressing expPABPN1 with ADAPT-232 resulted in markedly increased cell survival compared to non-treated cells (Figure 4D and E). Interestingly, this increase was accompanied by the increased expression of heat shock protein level in ADAPT-232 treated cells (Figure 4F).

The ubiquitin-dependent 26S proteasomal degradation process is the major system that mediates the degradation of damaged and short-lived regulatory proteins [67]. This active, ATP-dependent pathway involves a cascade of three types of enzymes that tag substrate proteins with an ubiquitin chain, for their recognition and subsequent proteolysis by the 26S proteasome. Proteasomes, the primary mediators of ubiquitin–protein conjugate degradation, are regulated through complex mechanisms. USP14, a proteasome associated deubiquitinating enzyme, can inhibit the degradation of ubiquitin–protein conjugates, in vivo and in vitro. Proteasomal activation is expected to demonstrate clinical utility, considering that oxidatively damaged proteins and protein aggregates are the cause of a large number of neurological diseases [68]. A recent Nature study showed that treatment of cultured cells with USP14 inhibitor enhanced degradation of several proteasome substrates that have been implicated in neurodegenerative diseases [69]. We hypothesized that the enhancement of proteasome activity through the inhibition of USP14 could offer a strategy to reduce the levels of aberrant expPABPN1 in cells. Therefore, we tested USP14 inhibitor for its protective effects on our established C2C12 stable transfecant model. Our results indicate that USP14 inhibitor indeed confers protection from cell death associated with expPABPN1 (Figure 4D). Our results highlight the importance of chaperone proteins and ubiquitination in OPMD pathology and have opened up...
broadened the spectrum of potential therapies for OPMD to include ADAPT-232 and USP14 inhibitor.

**Screens for drug discovery in OPMD (high-throughput screening)**

PABPN1 nematodes are useful to identify active compound families. *C. elegans* has several characteristics that make it ideal for drug testing including: a short lifecycle; small size; and the ease of culturing in liquid medium [70]. Furthermore, decades of neurobiological drug studies in *C. elegans* provide a strong foundation for use of this organism in therapeutic compound identification. The *C. elegans* muscular degeneration model has been used to evaluate the efficacy of pharmacological treatments on muscle function in OPMD [16]. Recently, our collaborator conducted a high-throughput drug-screening (over 2000 small molecules) in OPMD nematodes based on results from automated whole-animal fluorescence-based imaging. A screen for the small molecules that reverse PABPN1 toxicity found six promising drug candidates. To investigate the effects of these drugs in mammalian cells, we have recently tested these six drugs in C2C12 OPMD model and confirmed their protective effects in cells expressing PABPN1. To estimate the effect of the six compounds on the survival of model cells, we analyzed the cell survival using our established method of automated fluorescent microscopy [18]. In addition to increasing the cell survival, these drugs also enhanced muscle differentiation. Each drug's treatment showed consistent and significant protective effects against GFP-PABPN1-17Ala induced cell death, compared to the non-treated counterparts at different time points (data not shown). We looked at nuclear morphology to determine cell viability, and GFP-expressing cells with fragmented or condensed nuclei were counted as dead. While increased cell death and a progressive loss of green living cells was present in all cells expressing GFP-PABPN1-17Ala over the time course, each drug significantly increases the number of living cells (data not shown). Notably, these compounds were employed in the concentration of 10 μM without any cytotoxic effect. Further work needs to be done to determine the mechanism in which these drugs confer their effects in OPMD. Due to the efficiency of conducting screens in cell culture, and the increased physiological relevance of *C. elegans*, this methodology represents a powerful approach to identify candidate drugs for OPMD treatment.

**Antisense oligonucleotides: RNA replacement therapy**

The combination of the molecular knockdown of disease protein with the restoration of wild-type activity represents a promising strategy for the treatment of dominant diseases such as OPMD. The development of therapies for OPMD benefits from a certain characteristic of the disease: it is caused by a defect in a single known gene. In theory, agents that reduce the level of the mutant gene should alleviate the disease. Such reduction might be achieved using antisense oligonucleotides (ASOs) that target mRNA and inhibit expression of the disease gene [71]. ASOs are potentially potent and selective agents. ASOs can alter target gene expression by binding to RNA. Once bound, the ASO either disables or induces the degradation of the target RNA. Recent clinical trials confirm the ability of antisense oligonucleotides to significantly suppress target-gene expression [72, 73]. It is known that most OPMD patients are heterozygous at the OPMD locus, carrying one mutant and one wild-type allele [4]. Although mutant PABPN1 protein is toxic to muscles, its wild-type counterpart is protective against apoptotic muscle death [74]. An effective gene therapy must silence the deleterious mutant allele without eliminating expression of the beneficial wild-type one.

Our second OPMD treatment strategy is RNA replacement therapy. The goal is to develop RNA molecules (e.g., hammerhead ribozymes or hhRzs) capable of cleaving the mutant PABPN1 transcript and simultaneously replacing it with a normal, (possibly codon-optimized) transcript resistant to knockdown by the same ribozymes. Recently, we presented a new publicly accessible web-service, RiboSoft, which implements a comprehensive hammerhead ribozyme design procedure [75]. Inputting a target sequence into the software generates a set of ranked hammerhead ribozymes targeting the input sequence. Using this software, we were able to assay and validate four ribozymes targeting PABPN1’s transcript. These four ribozymes were successfully tested in vitro and in vivo, for their ability to cleave the targeted transcript [75]. Based on our promising results, we were encouraged to pursue this strategy and optimize the relevant therapy for OPMD. As the mutation in OPMD is a very small expansion of GGC within an alanine tract, these RNA molecules cannot distinguish between wild type and mutant PABPN1 RNAs. Introduction of the molecule into cells would cause the cleavage and degradation of all endogenous PABPN1 transcripts. Therefore, we decided to design and use an optimized-codon wild-type PABPN1 that is resistant to cleavage by hhRzs (i.e. opt-resistant PABPN1). Our idea is to introduce one or more hhRz and opt-resistant PABPN1 simultaneously to cells to both silence the mutant PABPN1 and replace it with wild-type PABPN1. For this purpose, we first validated the efficiency of our designed hhRzs (previously tested in vitro [75]) in their capacity to target and knockdown the transcript of PABPN1 in mammalian cells (Figure 5A). Our results identified highly effective hhRzs potent in knocking down PABPN1 (Figure 5A). Interestingly, our in vivo results confirmed the efficiency of the previously effective hhRzs in vitro [75]. Second, we designed the opt-resistant PABPN1 and tested its expression in cells (Figure 5B). We then co-transfected hhRzs and opt-resistant PABPN1 in cells and observed the level of PABPN1 expression. Transfection of opt-resistant PABPN1 restored PABPN1 expression from hhRs silencing as shows by Western blot (Figure 5B). These results indicate that opt-resistant PABPN1 is resistant to cleavage by hhRs. The testing of
these hhRzs with opt-resistant PABPN1, as a form of RNA replacement therapy, is currently underway in different OPMD cell and animal models.

Gene editing: CRISPR-Cas9

Muscle regenerative stem cells remain an attractive option for the treatment of muscle disorders. Muscle stem cells have unique properties that are not restricted solely to the formation of new muscle, but also contribute to the repair of damaged residual tissue [76].

Clustered regularly interspaced palindromic repeats (CRISPR)-associated (Cas) system is a powerful genome editing tool consisting of the Cas9 nuclease and a single guide RNA (sgRNA). The sgRNA targets Cas9 to genomic regions that are complementary to the 20-nucleotide (nt) sgRNA sequence and contain a 5’-NGG-3’ protospacer-adjacent motif (PAM). Double-stranded DNA breaks generated by Cas9 at target loci are repaired by non-homologous end-joining or homology-directed repair (HDR) [77]. Unlike conventional methods, which either temporarily address disease symptoms or randomly integrate therapeutic factors in the genome, CRISPR-Cas9 is capable of correcting the underlying cause of the disease, therefore permanently eliminating the symptoms with precise genome modifications [77]. Several new experiments published in Science demonstrate that we can now edit the genomes of mice with muscular dystrophy, effectively restoring a large portion of function in the heart and other muscles. These experiments show exciting potential for the use of CRISPR in mammalian gene editing, which could eventually lead to cures for this and other genetic diseases in humans [78-80]. The strategy of combined genetic repair with cellular reprogramming to generate stem cells capable of muscle regeneration could be applied for treatment to other muscle dystrophies caused by single gene defect like Duchenne muscular dystrophy and OPMD [81] (Figure 6A). Using this method, it is would be advantageous to predetermine the edited DNA sequence in isolated cells from OPMD patients in advance and then, only after proving the gene correction was successful, transplant the cells back to the patient (Figure 6A).

Using a variety of available resources including transgenic OPMD models [36] and OPMD patient fibroblasts, we are currently investigating the potential of CRISPR-Cas9-mediated genome editing approach for correcting the mutation in OPMD as a third strategy for therapy. The primary objective is to correct the genetic defect in the PABPN1 gene of OPMD mice by CRISPR/Cas9-mediated genome editing. Our ultimate goal is to translate this genome editing technology (cleavage and replacement of PABPN1 gene) into a novel class of human therapeutics for OPMD patients.

Simply, the sgRNA-Cas9 complex targets PABPN1 specifically through RNA-DNA base pairing, allowing Cas9 to...
cleave the DNA. The use of two sgRNAs targeted to the 5' and 3' ends of the \textit{PABPN1}, permits the complete excision of the gene. As \textit{PABPN1} gene is mutated in OPMD, we will stimulate the repair by adding a normal copy of \textit{PABPN1} gene to the cell. The ends of this normal copy will be further differentiated into myoblasts to form myofibers. Either myoblasts or myofibers can be transplanted to patients, but only for transient recovery, as myoblasts or myofibers will eventually die after cellular turnover. An ideal approach would be to differentiate iPS cells into satellite cells, which are muscle stem cells, to gain long-term self-renewal and regeneration capacity in the myofibers. Currently, ex vivo expansion of primary satellite and genome editing is challenging, but progress here could circumvent the use of iPS cells. Reprinted from Genome Editing Gene Therapy for Duchenne Muscular Dystrophy, Hotta, Akitsu. Journal of Neuromuscular Diseases, vol. 2, no. 4, pp. 343-355, Copyright (2015), with permission from IOS Press.

Figure 6A. Ex vivo gene therapy approaches using iPS cells. A scheme for induced pluripotent stem cells (iPS) cell-mediated ex vivo gene therapy approaches for DMD. Skin fibroblasts or monocytes from peripheral blood are reprogrammed to iPS cells by transient expression of the Yamanaka factors. The dystrophin mutation can then be repaired using genome engineering technologies. Corrected iPS cells can then be further differentiated into myoblasts to form myofibers. Either myoblasts or myofibers can be transplanted to patients, but only for transient recovery, as myoblasts or myofibers will eventually die after cellular turnover. An ideal approach would be to differentiate iPS cells into satellite cells, which are muscle stem cells, to gain long-term self-renewal and regeneration capacity in the myofibers. Currently, ex vivo expansion of primary satellite and genome editing is challenging, but progress here could circumvent the use of iPS cells. Reprinted from Genome Editing Gene Therapy for Duchenne Muscular Dystrophy, Hotta, Akitsu. Journal of Neuromuscular Diseases, vol. 2, no. 4, pp. 343-355, Copyright (2015), with permission from IOS Press.

Competing interests

The authors declare no conflict of interest.

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