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Progressive myopathy in an inducible mouse model of oculopharyngeal muscular dystrophy

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Abstract

The genetic basis of oculopharyngeal muscular dystrophy (OPMD) is a short expansion of a polyalanine tract (normal allele: 10 alanines, mutant allele: 11–17 alanines) in the nuclear polyadenylate binding protein PABPN1 which is essential for controlling poly(A) tail length in messenger RNA. Mutant PABPN1 forms nuclear inclusions in OPMD muscle. To investigate the pathogenic role of mutant PABPN1 *in vivo*, we generated a ligand-inducible transgenic mouse model by using the mifepristone-inducible gene expression system. Induction of ubiquitous expression of mutant PABPN1 resulted in skeletal and cardiac myopathy. Histological changes of degenerative myopathy were preceded by nuclear inclusions of insoluble PABPN1. Downregulation of mutant PABPN1 expression attenuated the myopathy and reduced the nuclear burden of insoluble PABPN1. These results support association between mutant PABPN1 accumulation and degenerative myopathy in mice. Resolution of myopathy in mice suggests that the disease process in OPMD patients may be treatable.

Keywords

Oculopharyngeal muscular dystrophy; transgenic mouse model; PABPN1; myopathy; cardiomyopathy; protein aggregation; nuclear inclusion

Introduction

Oculopharyngeal muscular dystrophy (OPMD) is caused by expansion of a polyalanine tract at the N-terminus of the nuclear polyadenylate binding protein PABPN1 from its normal length of 10 alanines (A₁₀) to its pathological length of 11–17 alanines (A₁₁–A₁₇) (Brais et al., 1998). OPMD is an adult-onset progressive skeletal myopathy that involves the ocular, oropharyngeal, and limb muscles. Filamentous inclusions in muscle nuclei are a pathological hallmark in OPMD (Becher et al., 2000; Calado et al., 2000; Tome and Fardeau, 1980;

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Conflict of Interest Statement
None declared

Uyama et al., 2000). However, the exact role of the PABPN1 inclusions in disease pathogenesis is not yet defined. Initial research on various cellular models suggested that the expanded polyalanine tract predisposed to PABPN1 aggregation and promoted cell death (Abu-Baker et al., 2003; Bao et al., 2004; Brais, 2009; Fan et al., 2001). More recent studies have instead suggested that large PABPN1 aggregates may be bystanders of PABPN1 toxicity or might even be protective (Brais, 2009; Messaëd et al., 2007).

Transgenic mouse models of OPMD have been generated using different promoters and polyalanine tract lengths. Ubiquitous expression of mutant PABPN1 (A₁₃) in transgenic mice led to growth retardation and premature death (Hino et al., 2004). Early lethality in these mice may have resulted from toxic effects of mutant PABPN1 on non-muscle tissues during development. Expression of mutant PABPN1 (A₁₇) using its natural promoter in mice led to a neuronal phenotype with a surprising absence of myopathy (Dion et al., 2005). Notably, a CNS disease characterized by cognitive decline and psychotic manifestations was reported in individuals who are homozygous for the OPMD mutation (Blumen et al., 2009). These studies suggest that the disease process in OPMD can extend beyond skeletal muscle. Finally, muscle specific expression of PABPN1 (A₁₇) results in myopathy with prominent apoptosis in mice, a feature not reported in human disease (Davies et al., 2005). Treatment of transgenic mice with the chemical chaperone trehalose or the anti-amyloidogenic agent doxycycline attenuated the muscle disease (Davies et al., 2006; Davies et al., 2005); however, both agents have antiapoptotic properties, and therefore therapeutic effects may be related to inhibition of cell death pathways independent of PABPN1 aggregation.

Despite important contributions of these transgenic mouse models to advancing our understanding of disease mechanisms in OPMD, the following questions remain: (1) is the progressive nature of the disease process in OPMD dependent on continuous expression of mutant PABPN1?; and (2) is it feasible to reverse the disease process by stopping the expression of mutant PABPN1? To address these questions, we developed a ligand-inducible transgenic mouse model of OPMD using the mifepristone (MFP) inducible gene expression system (Wang et al., 1994). Previous studies in mice have shown that this system is effective in regulating the expression of target transgenes in liver (Pierson et al., 2000; Wang et al., 1997), lungs (Zhao et al., 2001) and epidermis (Wang et al., 1999). This bitransgenic system consists of a target transgene and a second transgene encoding the engineered transcription factor GLVP which is activated by synthetic anti-progestins, such as MFP, but not by endogenous steroids. Levels of MFP required for activation are very low and do not have other known physiologic effects (Wang et al., 1994). The DNA binding domain of GLVP is derived from the yeast GAL4 gene, and is not known to bind or regulate endogenous mouse genes (Wang et al., 1997). Compared to tetracycline-regulated systems, a potential advantage of the MFP-inducible gene expression system is its rapid kinetics of induction and de-induction. For example, a transgene expressed in liver was maximally induced within 12 hours after intraperitoneal (i.p.) injection of MFP, and returned to basal levels within 86 hours (Wang et al., 1997). Using this bitransgenic system, we found that MFP-inducible ubiquitous expression of mutant PABPN1 produced a progressive skeletal myopathy and dilated cardiomyopathy.

Material and methods

Transgene constructs

The following transgene constructs were prepared for microinjections: 1) GAL4-tk-PABwt; 2) GAL4-tk-PABmut; 3) GAL4-tk-luciferase; and 4) CAG-GLVP. A 302 bp fragment consisting of four copies of the GAL4 consensus binding sequence linked to the thymidine kinase gene promoter (tk) was derived from 17x4-tk-CAT (Wang et al., 1994) (received from Drs. Sophie Tsai and Bert O'Malley, Baylor College of Medicine, Houston, TX). The

modified human PABPN1 minigene (PABwt; A₁₀) was prepared by adding the following sequences to human PABPN1 cDNA: 1) a 28 bp 5' untranslated region (UTR) of the human skeletal muscle alpha actin gene; 2) optimized translational initiation (Kozak) signal; 3) a codon-substituted polyalanine tract; and 4) sequences of intron 2, and intron 3 of human *PABPN1* gene. Intron 1 was not included because retention of this intron is a potential mechanism for regulating synthesis of PABPN1 protein. The *PABPN1* 3' UTR was replaced with the human growth hormone 3' UTR because PABPN1 could potentially autoregulate its own mRNA by interacting with the highly conserved internal polyadenine runs in the 3'UTR. For the mutant PABPN1 construct (PABmut; A₁₆), linkers were used to insert additional six alanine codons to the polyalanine tract in the PABwt. The PABwt was replaced with luciferase cDNA to generate the GAL4-tk-luciferase. For the CAG-GLVP construct, GLVPc914 cDNA (Wang et al., 1999) (received from Drs. Sophie Tsai and Bert O'Malley) was inserted in pCAGGS plasmid (Niwa et al., 1991) (received from Dr. J. Miyazaki, Osaka University, Osaka, Japan). All constructs were sequenced to confirm that the polyalanine tract was not interrupted, and that the PABPN1 exons and introns as well as the luciferase and GLVP coding regions were intact.

Transgenic mice

PABmut (n=4), PABwt (n=2), and luciferase (n=1) lines were derived on the FVB inbred background. GLVP lines (n=3) were derived on the C57BL/6 inbred background. Wild-type, single transgenic PABPN1 or luciferase, and btransgenic GLVP/PABPN1 or GLVP/luciferase mice were F1 hybrids of crossing PABPN1 or luciferase with GLVP transgenic lines. Mice were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Animal experiments were approved by the University Committee on Animal Resources at the University of Rochester Medical Center. For acute induction, mice were treated with intraperitoneal (i.p.) injections of 1 mg/kg MFP daily in two divided doses for 4 days. MFP was initially dissolved in dimethyl sulfoxide at a concentration of 40 mg/ml, and the working solution was prepared in physiological saline with 0.01% Triton-X at a concentration of 1mg/ml. Chronic induction was achieved by serial subcutaneous implantation of custom made slow-release pellets (Innovative Research of America) that deliver 1 mg/kg/day MFP or placebo for up to 8 weeks. Littermates were randomly selected for MFP or placebo and the investigators were blinded to randomization. For rotarod performance, automated recording and multiple lanes allowed for simultaneous analysis of mice in different experimental groups on the rotarod (Columbus Instruments). The mice were individually trained on the rotarod at 10 rpm speed for a maximum of 1 minute duration. The mice were given 2 such training sessions per day with at least 5 minutes of rest between the sessions for two consecutive days. After this training phase, the time to fall was measured during 3 consecutive trials at 10 rpm and 20 rpm speed with 20s inter-trial rest. The longest time on the rotating drum was recorded. The maximum score was 200 seconds. These measurements were repeated for 3 consecutive weeks and the average performance was used in comparison between the experimental groups.

Competitive RT-PCR assay

Total cellular RNA was isolated (TriReagent; Molecular Research Center) and first strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) with oligodT₍₁₂₋₁₈₎. A competitive RT-PCR assay normalized to equal cDNA input was performed as described (Welle et al., 2000) to measure relative amounts of transgene mRNA. The following primers were used to generate the internal standard: forward 5' - GGCTGGTGGGCACTGGAG -3', and reverse 5' - CAGACCAGGCATCAGCACAACGCTCTCGATTCTACAG -3'. The forward primer and the underlined priming sequence in the reverse primer were used to amplify a 284bp fragment of the transgene cDNA and the 231 bp internal standard in each tube. The PCR

products were resolved on agarose gels, stained with SybrGreenII (Molecular Probes), and analyzed on a laser fluorimager.

Allele-specific RT-PCR assay

There is >90% homology between murine and human PABPN1 allele. A species difference creates a BanII restriction site at nucleotide 2074 in the human PABPN1 mRNA (GenBank accession no. NM_004643) that does not exist in the murine homologue. The following primers were perfectly complementary to human and murine cDNAs: forward at 2033bp, 5' –AGACCAGGCATCAGCACAAAC –3', and reverse at 2193bp, 5' –GAATACCATGATGTCGCTCTAGC – 3'. BanII digestion of the RT-PCR products generated a 160 bp fragment from the murine transcript, and 119bp and 41bp fragments from the transgenic mRNA. The digested products were resolved on agarose gels, stained with SybrGreenII (Molecular Probes), and analyzed on a laser fluorimager.

Histology, immunofluorescence, and electron microscopy

Frozen 10 µm sections of skeletal muscles and paraffin embedded sections of hearts and brains were stained by standard histologic methods. Immunofluorescence was performed on adjacent 6 µm sections of frozen tissues using anti-PABPN1 polyclonal antibody (1:500 dilution) in the presence or absence of 1M KCl (Calado et al., 2000). The anti-PABPN1 antibody was made against the peptide NH₂-RSGSPGRRRHLVPGAGGEC-COOH (D.G. Bear and M.W. Becher, University of New Mexico, Albuquerque, unpublished results; and (Sagawa et al., 2011)). For morphometry, frozen muscle sections were stained with anti-laminin antibody (1:80 dilution) (Sigma) to delineate basement membrane of the muscle fibers as described previously (Mankodi et al., 2000). Secondary antibodies were conjugated either with Alexa 488 or Alexa 680 (1:400 dilution). Muscle fiber cross-sectional areas were measured using ImageProPlus v3.0. Standard procedures were used to prepare mouse tissues for electron microscopy.

Filter trap assay and immunoblot

For the filter trap assay (Scherzinger et al., 1997; Wanker et al., 1999), protein samples were heated at 98°C for 3 min in 2% SDS with 2M NaCl, and filtered through a 0.2 µm cellulose acetate membrane (Whatman) using a Gibco-BRL dot-blot filtration unit. The membranes were probed with the anti-PABPN1 polyclonal antibody (1:2000 dilution). Secondary antibody was conjugated with either IRDye680 or IRDye800 (1:10,000 dilution) (LI-COR). The membranes were imaged using Odyssey infrared imaging system (LI-COR). Immunoblots were probed with the anti-PABPN1 polyclonal (1:2000 dilution), anti-GAPDH monoclonal (1:5000 dilution) (Sigma) or anti-GAL4 polyclonal (1:400 dilution) (Santa Cruz) antibodies.

Luciferase assay

Luciferase activity was measured using kits (Luciferase assay system, Promega) according to the manufacturer's instructions.

Results

Generation of PABPN1 and GLVP transgenic mice

PABPN1 minigenes were generated with 10 alanines (PABwt) or 16 alanines (PABmut) (Fig. 1A). To enhance control over PABPN1 induction, human PABPN1 minigene was designed to disable potential autoregulatory mechanisms and increase the translational efficiency of the transgene mRNA (Supplementary Fig.1A; see methods). Expression of the minigene was controlled by a minimal thymidine kinase (tk) promoter in series with four

GAL4 response elements (Wang et al., 1994) (Fig. 1A). The expression of GLVP was driven by the CMV early enhancer/chicken beta actin (CAG) promoter (Niwa et al., 1991) (Fig. 1A). PABmut (n=4) and PABwt (n=2) transgenic lines were derived on the FVB inbred background. GLVP transgenic lines (n=3) were derived on the C57BL/6 inbred background. GLVP/PABwt and GLVP/ PABmut bitransgenic mice were F₁ hybrids resulting from the cross between GLVP and respective PABPN1 transgenic lines.

Induction of PABPN1 expression in GLVP/PABwt and GLVP/PABmut bitransgenic mice

To test the ability of GLVP transgenic lines to support MFP-regulable gene expression, we bred mice in each of the three GLVP transgenic founder lines with the GAL4-tk-luciferase reporter mice (Supplementary Fig. 1B). In the GLVP line that showed optimal induction, 4 days of i.p. MFP injection (1mg/kg/day) caused a >10,000-fold increase of luciferase activity in muscle, heart, and brain (Supplementary Fig. 1C). Implantation of subcutaneous timed-release MFP pellets (1mg/kg/day; Innovative Research of America) also caused a sustained induction of luciferase activity in tissue (data not shown). This line of GLVP transgenic mice was used in subsequent experiment for PABPN1 induction.

In previous studies the GAL4-tk element did not drive detectable gene expression of reporter genes in single transgenic mice (Schmidt et al., 1990; Wang et al., 1997). In contrast, we found low basal levels of PABmut mRNA in muscle (Supplementary Fig. 1D), heart, and brain (data not shown) in each of the four PABmut founder lines. However the expression levels were lower than endogenous Pabpn1 mRNA, as determined by an allele-specific RT-PCR assay (Supplementary Fig. 1D), and did not result in histopathologic changes in these organs in any of the PABmut founder lines (n=5 mice examined in each line).

Next, we bred transgenic mice in the “optimal” GLVP founder line with each of the PABPN1 transgenic mice and selected 3 PABmut (Pabmut -A, -E, and -G), and one PABwt lines that showed highest induction. By a competitive RT-PCR assay, we observed a 2-3 - fold induction of PABmut (Fig. 1B) or PABwt mRNA (data not shown) in skeletal muscle, heart, and brain after 4 days of i.p. MFP-injection (1mg/kg/day). The induction of PABmut mRNA (Fig. 1C) or PABwt mRNA (data not shown) appeared similar when measured relative to endogenous murine PABPN1 using the allele-specific RT-PCR assay. By serial implantation of subcutaneous timed-release MFP pellets (1mg/kg/day) beginning at age 2 months, we maintained a 3-4 fold induction of PABPN1 mRNA for periods of several months in GLVP/PABmut bitransgenic mice (Fig. 1D) and GLVP/PABwt bitransgenic mice (Supplementary Fig. 2A). Immunoblots showed a 5-10 -fold induction of PABPN1 protein at 3 weeks in GLVP/PABmut mice in skeletal muscle (Fig. 1E), heart (Supplementary Fig. 1E), and brain (data not shown), with further accumulation of PABmut protein in muscle tissue during chronic induction (Fig. 1E). Similar levels of normal PABPN1 protein induction was seen in skeletal muscle (Supplementary Fig. 2B), heart, and brain (data not shown) in MFP-treated GLVP/PABwt mice.

Induction of mutant PABPN1 leads to a progressive myopathy in GLVP/PABmut bitransgenic mice

GLVP/PABmut bitransgenic mice in 3 different PABmut founder lines developed a consistent phenotype when induced by MFP (n=9-13 mice examined in each founder line). The first signs to appear were reduced cage activity and grooming behavior around 10-12 weeks after the first MFP pellets were implanted. At this stage 13% of the induced GLVP/ PABmut mice died suddenly; post-mortem examination showed dilated cardiomyopathy. Subsequently mice developed progressive muscle wasting, and thoracic spine kyphosis (Fig. 2A). Rotarod performance was markedly impaired (time to fall at 10 rpm 7-14 sec versus >3min for placebo-treated mice) (Fig. 2B). More than 70% of mice died or were euthanized

for humane reasons within 10 months of induction, and none survived beyond 12 months (Fig. 2C). In contrast, placebo-treated GLVP/PABmut mice (n=15) and PABmut single transgenic mice (treated with MFP; n=12) had normal survival and cage behavior. None of the MFP-treated GLVP/PABwt mice showed behavioral change (n=10). However, three of these mice died after 4 -10 months of induction (Supplementary Fig. 2C). Necropsy studies did not show any organ pathology or reveal the cause of death in these mice. Placebo-treated GLVP/PABwt mice (n=5) and PABwt single transgenic mice (treated with MFP, n=3) showed normal survival to age 14 months.

We examined muscle histology after MFP-induction of PABmut expression. Muscle histology was unremarkable at 3 weeks after induction (Fig.2D). After 6 months of induction, GLVP/PABmut muscles showed internal nuclei, variability of muscle fiber size, and increased connective tissue (Fig. 2E). Placebo-treated mice had normal muscle histology. Relative to placebo-treated mice, MFP-treated GLVP/PABmut mice showed atrophy of muscle fibers (Fig. 2F). Average muscle fiber area was $1270 \pm 100 \mu\text{m}^2$ in MFP-treated GLVP/PABmut mice and $1930 \pm 180 \mu\text{m}^2$ in placebo-treated GLVP/PABmut mice (n=3 each, 300 fibers each muscle section). As in human OPMD, we observed rimmed vacuoles in cytoplasm of some muscle fibers (inset in Fig. 2E). There was little evidence of muscle fiber necrosis or inflammation. Muscle disease was widespread with the involvement of pharyngeal, diaphragm, paraspinal and limb muscles. In contrast, muscle histology was normal in MFP-treated GLVP/PABwt mice (Supplementary Fig. 2D). Semithin sections of vastus muscle revealed abnormally enlarged myonuclei containing clear zones within the nucleoplasm in MFP-treated GLVP/PABmut mice (Fig.2G). Ultrastructural studies showed large nuclear collections of filamentous structures (Fig. 2H-I). In contrast to human OPMD (Tome and Fardeau, 1980) and similar to previously reported mouse model of OPMD (Davies et al., 2005; Hino et al., 2004), the filamentous structures did not form well defined palisades or tangles. Similar filamentous structures were not observed in either MFP-treated GLVP/PABwt mice or placebo-treated GLVP/PABmut bitransgenic mice.

MFP-treated GLVP/PABmut mice, but not placebo-treated bitransgenic littermates, developed dilated cardiomyopathy with disarray of cardiomyocytes, cellular infiltrates, and increased connective tissue (Fig. 2J-K). Cardiac enlargement was seen as early as 3 months after induction. Hearts were three to five times heavier than age and sex matched wild type and placebo-treated bitransgenic mice after 6-10 months of induction (Fig. 2L). Although GLVP transactivator was expressed at a higher level in heart than skeletal muscle (Supplementary Fig. 1F), the cardiomyopathy was not caused by GLVP itself as none of the MFP-treated GLVP mice developed cardiac enlargement (data not shown). In addition, the MFP-treated GLVP/PABwt bitransgenic mice did not show cardiomyopathy (Supplementary Fig.2E).

Whereas mutant PABPN1 mRNA was induced in brain to levels matching skeletal and cardiac muscle (Fig. 1B), brain histology remained normal in all areas examined in MFP-treated GLVP/PABmut bitransgenic mice (Supplementary Fig. 3A-B).

Accumulation of insoluble PABPN1 protein precedes the onset of disease in MFP-treated GLVP/PABmut bitransgenic mice

A previous study of OPMD showed that mutant PABPN1 protein formed inclusions that were resistant to extraction in high salt buffers (Calado et al., 2000). To examine the solubility state of PABPN1 in our model we prepared frozen sections of tissue, extracted with 1M KCl for 5 minutes, and then detected residual PABPN1 with polyclonal antibodies. PABPN1 (transgene-encoded plus endogenous) was readily extracted in wild type muscle (Fig. 3A-B), heart, and brain and in these tissues in placebo-treated GLVP/PABmut mice. In contrast, in MFP-treated GLVP/PABwt tissues, occasional nuclei showed deposition of

PABPN1 that resisted extraction in 1M KCl (Supplementary Fig. 2F). However, the inclusions of insoluble PABPN1 were more frequent and conspicuous in skeletal muscle, heart, and brain of MFP-treated GLVP/PABmut mice, occurring as early as 3 weeks after induction, when mice showed no behavioral change and histology was still normal (Fig. 3C; data shown in skeletal muscle). As the disease advanced to later stages, most nuclei had massive accumulations of insoluble PABPN1 in skeletal muscle (Fig. 3D), heart (Fig. 3E), and brain (Supplementary Fig. 3C-D).

We also used a filter trap assay (Scherzinger et al., 1997; Wanker et al., 1999) to confirm a change in the solubility state of PABPN1 protein. In MFP-treated GLVP/PABmut bitransgenic mice, the alanine-expanded protein in skeletal muscle (Fig. 3F), heart (Fig. 3G), and brain (Supplementary Fig. 3E) was insoluble in 2% SDS and retained on a 0.2 μ m cellulose acetate filter. SDS-insoluble PABPN1 was detected in small amounts before the onset of disease (Fig. 3F; data shown in skeletal muscle). The amount of insoluble PABPN1 protein increased with the duration of induction (Fig. 3F). The filter trap assay did not detect SDS-insoluble PABPN1 in wild type or MFP-treated GLVP/PABwt mice (data not shown) or single transgenic or placebo-treated mice (Fig. 3G).

Attenuation of myopathy and disappearance of insoluble PABPN1 after down-regulation of PABmut expression in GLVP/PABmut bitransgenic mice

To investigate reversibility of the disease process in MFP-treated GLVP/PABmut mice, MFP was withdrawn after 4 months of treatment (OFF mice, n=4 in the GLVP/PABmut-E line), a duration of induction that caused 50% lethality in this bitransgenic line (Fig. 2C). Comparisons were made to GLVP/PABmut littermates that were sacrificed for analysis after 4 months of MFP-induction (ON mice, n=5). As expected, there was a 4-5 fold induction of PABmut mRNA in ON mice, whereas PABmut mRNA levels were closer to endogenous Pabpn1 mRNA in OFF mice (Fig. 4A). OFF mice began to show signs of behavioral improvement within 3-4 weeks of MFP withdrawal. Specifically cage behavior improved, and there was better rotarod performance than ON mice (Fig. 4B). OFF mice never developed signs of major impairment and survived beyond the lifespan of any MFP-treated GLVP/PABmut mice in cohort. In contrast with ON mice, the hearts in OFF mice did not appear enlarged and weighed closer to wild type hearts (Fig. 4C). Different aspects of myopathic changes including increase in internal nuclei, and connective tissue and muscle fiber atrophy seen in the ON mice were mild or absent in OFF mice (Fig. 4D). Myonuclei had a heavy load of 1M KCl-insoluble PABPN1 in ON mice, whereas only occasional nuclei showed small punctate accumulations of PABPN1 in skeletal muscle (Fig. 4E), heart, and brain in OFF mice. The filter trap assay showed a similar reduction in the amounts of SDS-insoluble PABPN1 in OFF mice compared to ON mice (Fig. 4F).

Discussion

We used the MFP-inducible gene expression system (Wang et al., 1994) to generate a ligand inducible transgenic mouse model of OPMD. Inducible expression of mutant PABPN1 reproduced several aspects of skeletal myopathy seen in human OPMD. Particularly, the mice developed a progressive non-necrotic myopathy associated with rimmed vacuoles and myonuclear filamentous inclusions. Nuclear accumulations of KCl-insoluble PABPN1 were observed in skeletal muscle in the mice reminiscent of the human disease. Unexpectedly, inducible expression of PABmut in cardiac muscle also led to dilated cardiomyopathy in the mice. The heart disease was accompanied by nuclear accumulations of KCl-insoluble PABPN1 in cardiomyocytes as well as SDS-insoluble PABPN1 in the heart lysates. It is noteworthy that the cardiomyocyte-specific expression of an expanded polyglutamine repeat peptide (83Q) was sufficient to cause deposition of 83Q aggregates, cardiomyocyte death and dilated cardiomyopathy in transgenic mice (Pattison et al., 2008). Taken together these

observations suggest that misfolded proteins, independent of the peptide sequence, are directly toxic to cardiac muscle in mice. Further studies are needed to define the underlying cellular mechanisms by which accumulations of misfolded proteins lead to cardiomyocyte death and heart failure. Heart disease has not yet been reported in OPMD, which may be due to lower levels of mutant protein in human disease compared to the mice.

The ligand-inducible design for PABmut expression allowed us to define a time window between start of the mutant protein expression and onset of the clinical disease in contrast to conventional transgenic approaches used in previous mouse models (Davies et al., 2005; Dion et al., 2005; Hino et al., 2004). We observed that the onset of the clinical disease in MFP-treated GLVP/PABmut mice occurred after 2 months of continuous PABmut expression. This time-window provided a unique opportunity to characterize early events in disease pathogenesis in the mice. It has been established that expanded polyalanine tract influences mutant PABPN1 aggregation and toxicity in OPMD (Brais, 2009). During the pre-symptomatic stage when the histology was still normal in mice, we detected discrete nuclear inclusions of KCl-insoluble PABPN1 in skeletal muscle, heart, and brain as well as SDS-insoluble PABPN1 in the lysates from these organs. Soluble forms of mutant PABPN1 are considered to be toxic in cellular models (Messaed et al., 2007); however, in a chronic degenerative disease in mice it is difficult to separate relative toxicities of soluble oligomers and insoluble PABmut as the appearance of visible large aggregates inevitably involves the formation of soluble oligomers (Uversky, 2010). Whether the disease process is initiated by soluble oligomers or insoluble forms of PABmut, our results suggest that aggregation of PABmut is an early event and precedes the clinical and histological disease in the mice.

This MFP-inducible mouse model also allowed us to investigate whether the progression of the disease is dependent on continuous expression of PABmut or is reversible. Downregulation of PABmut expression at a stage when most mice had developed signs of skeletal myopathy, and dilated cardiomyopathy resulted in the attenuation of disease. We observed a significant decrease in the amounts of insoluble PABPN1 close to control levels indicating that the aggregation of PABmut was reversible in our mice. Previous studies with cellular models of OPMD suggested that PABPN1 aggregation directly impairs the function of ubiquitin-proteasome pathway as well as molecular chaperones (Abu-Baker et al., 2003); however, the disappearance of insoluble PABPN1 in the mice raises the possibility that disposal mechanisms for misfolded proteins may not be completely compromised in diseased tissues.

Overall, this inducible mouse model provides experimental evidence for the direct association between PABmut expression and myopathy. Importantly we show that silencing of PABmut expression reversed degenerative myopathy as well as accumulations of insoluble PABPN1 in mouse tissues. Our results suggest that silencing the expression of mutant PABPN1 may be an option for therapy in patients with OPMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- >We generated mifepristone-inducible transgenic mouse model of OPMD
- >Inducible expression of mutant PABPN1 resulted in skeletal and cardiac myopathy
- >De-induction of mutant PABPN1 attenuated the myopathy and reduced PABPN1 aggregates
- >Silencing the expression of mutant PABPN1 may be an option for therapy in OPMD

Figure 1. Inducible expression of PABmut transgene

(A). Diagram of GAL4-tk-PABwt (10 alanines), GAL4-tk-PABmut (16 alanines), and CAG-GLVP transgenes. The GAL4 binding domain of GLVP recognizes its cognate elements in the GAL4-tk-PABPN1 minigene. (B). PABmut mRNA levels in single transgenic PABmut mice (MFP-treated), or in vehicle (saline with 0.01% Triton X)- or MFP-treated (i.p.; 1mg/kg/day for 4 days) bitransgenic GLVP/PABmut mice (n=6, triplicate assay). Expression level was determined by competitive RT-PCR assay, normalized for equal cDNA input. There is a 2 to 3- fold increase in MFP-treated mice compared to vehicle-treated mice in vastus muscle ($P = 0.01$), heart ($P = 0.002$), and brain ($P = 0.004$). (C). Representative allele-specific PCR assay shows amount of PABmut mRNA (Tg.) relative to endogenous Pabpn1 mRNA (Endo.) in vastus muscle in vehicle- (n=3) and MFP-treated mice (n=2). Wild-type murine mRNA and normal human mRNA served as controls. Hum+mus mix denotes equal input of normal human and mouse muscle RNA into assay. Note that there is no preferential amplification of either allele. A representative gel of three independent experiments is shown. (D). Serial implantation of MFP pellets led to persistent increase in PABmut mRNA levels by 3- to 4-fold compared to placebo pellets after 3 weeks ($P=0.009$) and 6 months ($P=0.0006$) in vastus muscle in GLVP/PABmut mice. (E). Immunoblot for PABPN1 (transgene-encoded plus endogenous) in vastus muscle in wild-type mice (WT), placebo-treated or MFP- induced GLVP/PABmut mice (n= 3, triplicate assay). (Error bars $\pm$ S.E.M and unpaired t test). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2. Progressive myopathy in MFP-treated GLVP/PABmut bitransgenic mice
(A). Muscle wasting, splaying of the limbs and the toes, and thoracic spine kyphosis in MFP-treated mice. **(B).** Rotarod performance after 4 months of treatment with either placebo or MFP ($n = 9$; 10 rpm). **(C)** Survival analysis of MFP-treated mice [GLVP/PABmut A (purple), GLVP/Pabmut E (blue), and GLVP/PABmut G (yellow); $n = 9$ -13 mice in each line] and placebo-treated mice (black, $n = 5$ mice in each line). **(D-E).** Hematoxylin and eosin stained frozen vastus muscle sections show normal histology after 3 weeks of MFP-induction (D), but internal nuclei, variability in muscle fiber size, and increased connective tissue after 6 months (E); inset shows a cytoplasmic rimmed vacuole. **(F).** Frequency histogram of cross-sectional muscle fiber area shows muscle fiber atrophy in vastus muscle in MFP-treated mice relative to placebo-treated mice ($n = 3$, 300 fibers each, MFP-induction for 6 months). **(G).** Toluidine blue stained semithin sections of vastus muscle show clear zones within myonuclei (arrow) in MFP-treated mice. **(H-I).** Electron microscopy shows filamentous inclusions in myonuclei in vastus muscle in MFP-treated mice. **(J-K).** Hematoxylin and eosin stained heart sections show normal histology in placebo-treated mice (J). In contrast, there is increased cellularity and disorganization of cardiomyocytes in MFP-treated mice (K). **(L)** Relative size and weight of hearts (expressed as percent of body weight) in placebo- and MFP-treated mice. There is cardiomegaly and a 4-fold increase in heart weight in MFP-treated mice compared to placebo-treated mice ($n = 3$, F test, $*P = 0.01$), and no significant difference between placebo-treated and wild-type (WT) hearts (F test, $P = 0.17$). Scale bar 100 μm (E,K), 25 μm (inset in E), 5 μm (G), 1 μm (H), and 500 nm (I). The scale bar in E applies to D, and the scale bar in K applies to J. Error bars $\pm$ S.E.M (B,F,L).

Figure 3. Accumulation of insoluble PABPN1 in skeletal muscle and heart in MFP-treated GLVP/PABmut bitransgenic mice
 Immunofluorescence analysis of frozen sections using anti-PABPN1 antibodies. (A-B). Adjacent 6µm cross-sections of wild type (WT) vastus muscle show extraction of PABPN1 (green) from myonuclei (blue) by 1M KCl. (C-D). Representative images show progressive accumulation of 1M KCl insoluble PABPN1 in myonuclei in vastus muscle in MFP-treated mice. There are discrete nuclear inclusions after 3 weeks of induction, and massive amorphous nuclear accumulations after 6 months. (E). Myonuclear accumulations of 1M KCl insoluble PABPN1 (green) in frozen heart sections after 6 months of MFP-induction. (F-G). Representative images of filter trap assay show 2% SDS-insoluble PABPN1 aggregates in skeletal muscle (F), and heart (G) in MFP-treated mice. There is a progressive accumulation of SDS-insoluble PABPN1 from 3 weeks to 6 months after MFP-induction (F), and insoluble PABPN1 is not detected in placebo-treated mice (F). A representative blot of three independent experiments is shown. The scale bar 100 µm (C) applies to all panels.

Figure 4. Attenuation of myopathy and disappearance of insoluble PABPN1 after withdrawal of MFP in GLVP/PABmut bitransgenic mice

MFP was withdrawn after 4 months of continuous treatment in the GLVP/PABmut-E mice (OFF, n=4). Five of the littermates were euthanized to capture the snapshot of the disease at this stage (ON). **(A)**, PABmut mRNA levels relative to endogenous Pabpn1 mRNA in ON and OFF mice, determined by the allele-specific PCR assay. **(B)**, Rotarod performance in OFF mice at 4 months after withdrawal of MFP extended beyond 200s compared to <20s in ON mice (10rpm). **(C)**, Heart weights in OFF mice were similar to wild type (WT) hearts (F test, $P=0.12$), and lower than ON mice (F test, $**P=0.001$). **(D)**, Hematoxylin and eosin stained frozen vastus muscle sections show attenuation of various aspects of myopathy in OFF mice. Specifically, internal nuclei, and muscle fiber variability are mild or absent compared to ON muscle. **(E)**, Frozen vastus muscle sections stained with anti-PABPN1 antibody in the presence of 1M KCl. There are amorphous accumulations of PABPN1 (green) in myonuclei (blue) in vastus muscle in ON mice. In contrast, a majority of PABPN1 is extracted and only discrete inclusions remain in occasional myonuclei in OFF muscle. **(F)**, A filter trap assay shows reduction in the amounts of SDS-insoluble PABPN1 in skeletal muscle in OFF mice compared to ON mice. A representative blot of three independent experiments is shown. Scale bar 100 μm (D, E). Error bar (A-C) $\pm$ S.E.M.