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Nuclear Poly (A)-Binding Protein and Oculopharyngeal Muscular Dystrophy

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1. Introduction

Oculopharyngeal muscular dystrophy (OPMD) is an autosomal-dominant late-onset human genetic disease (Brais et. al, 1998). The symptoms usually appear around the age of fifty, and are characterized by drooping of the eyelid and swallowing difficulties. Both conditions may progress until the eyelid nearly or completely covers the eyeball (ptosis) and the ability to swallow is lost (dysphagia). In addition, patients suffer from proximal limb weakness; muscles of the shoulder and hip girdles may also gradually become weak. OPMD is highly prevalent amongst the French Canadian population of the Quebec province where almost one in every one thousand people is a carrier. In contrast only one in 100,000 people in Europe, including France is a carrier of OPMD. All cases of OPMD in Quebec could be traced to a single ancestor in the 15th century (Brunet et. al., 1990). OPMD is also more common amongst Bukhara Jews (Blumen et. al., 200). Possibly due to mass immigration during the 16th to 17th century, OPMD spread from Europe to many parts of the world (Hill et. al, 2001). OPMD patients have also been reported in Mexico, Thailand, Japan and China (Rivera et. al., 2008; Uyama et. al., 2000; Witoonpanich et.al., 2004; Ye et. al., 2011). A de novo germ line mutation has also been found in a Swiss OPMD patient (Gurtler et. al., 2006).

The mutation causing OPMD has been mapped to the gene encoding the nuclear poly (A) - binding protein PABPN1 at the short arm of chromosome 14 (14q11) of the human genome (Brais et. al., 1998). The human PABPN1 gene contains six GCG repeats following the AUG initiation codon. In OPMD patients expansion of the six GCG repeats to between 8-13 repeats have been found. A short poly alanines tract consisting of ten alanines is present at the N-terminal end of normal PABPN1. Six of these ten alanines are encoded by GCG while the last four alanines are coded by GCA. Compared to other trinucleotide expansion mutations such as the CAG expansion in Huntington's disease, the GCG expansion in OPMD is very modest and genetically stable. Mutations introducing two or more alanines are dominant whereas a single additional alanine expansion is recessive. Generally, the homozygous mutations exhibit more severe phenotypes than the heterozygotes. The severity of the disease increases with the increasing length of the GCG expansion, and also results in earlier onset of the disease (Messaed & Rouleau, 2009). The precise mechanism of trinucleotide repeat expansion in OPMD and other neurodegenerative diseases such as the

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Huntington is not clear. A slippage model, where the newly synthesized DNA strand dissociates and translocates to a new pairing position during DNA replication has been proposed. Perhaps this misalignment of the nascent strand in the repetitive tract results in the addition or deletion of repeats. Because of the stable nature of the GCG trinucleotide repeats of PABPN1 gene this model may not be applicable for the generation of mutation in OPMD patients. Unequal cross over during DNA replication may be the underlying mechanism for (GCG) repeat.

2. Structure and cellular function of PABPN1

Mammalian PABPN1 is a highly conserved nuclear RNA binding protein of 32.8 kDa with specificity for the poly (A) tract of eukaryotic mRNAs (Figure 1). It consists of one typical RRM domain with consensus RNP1 and RNP2 motifs in the central region of the polypeptide, separating the acidic glutamine rich N-terminal domain from the more basic arginine rich C- terminal domain (Kuhn et. al., 2003). The RNP domain and the C-terminal region of PABPN1 are required for binding to both RNA and its polypeptide partners. Interestingly the RNP domain of PABPN1 has no sequence similarity with the RNA binding domain of the cytoplasmic poly (A) - binding protein PABPC1 or other RNA binding proteins (Kuhn et. al., 2003). Recent crystal structure analyses of human PABPN1 suggest that PABPN1 RRM adopts a fold similar to canonical RRM structure consisting of a four stranded antiparallel β-sheet structure spatially arranged as β4β1β3β2. However, the fold of the third loop and dimerization of the crystal are distinct features of PABPN1 (Ge et. al., 2008).The nuclear localization signal is located between amino acids 289-306 and overlaps with the oligomerization domain (Abu-Baker et.al., 2005; Calado et.al., 2000). Due to the presence of the alanine tract PBPN1 is prone to aggregate formation. However, the polyalanine tract is not conserved, and is absent in Drosophila without any detectable loss of cellular function (Shinchuk et.al., 2005).
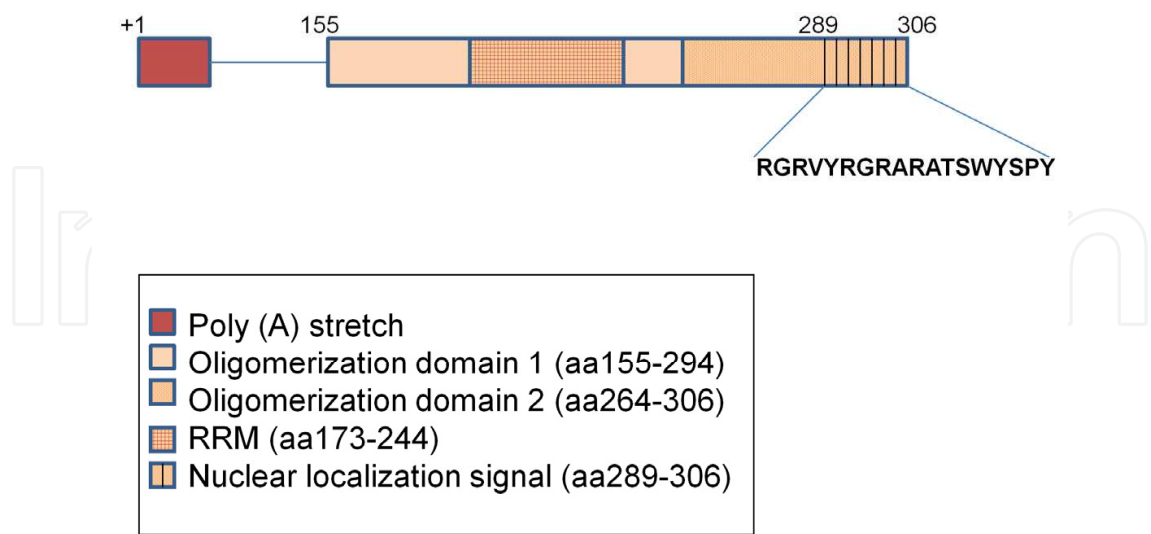

Fig. 1. Schematic diagram of various domains of PABPN1

The main cellular function of PABPN1 is to stimulate the elongation of poly (A) tract of eukaryotic mRNA, and at the same time control its length (Wahle, 1995). After the first ten adenine residues have been added PABPN1 binds to it as a monomer, and as the length of

the poly (A) tract increases additional PABPN1 assembles on the tract at a packing density of 15 adenines per PABPN1 molecule (Bienroth et. al., 1993; Kuhn & Wahle, 2004; Wahle, 1995). Both cleavage and poly adenylation specific factor (CPSF) and PABPN1 stimulate the activity of poly (A) polymerase by mutually stabilizing their interaction with mRNA in a transient complex. Although both CPSF and PABPN1 alone can stimulate the polyadenylation by poly (A) polymerase but the extension of the 3` end is much faster when both are present. When the poly (A) tail length has reached 250-300 nucleotides, further extension of the poly (A) tract becomes very slow (Wahle, 1995). The oligomerization of PABPN1 is functionally important and may serve as a molecular ruler to determine the length of the poly (A) tract (Keller et. al., 2000). The wild type PABPN1 exists in equilibrium as monomers, dimmers and oligomers and filamentous complexes (Nemeth et. al., 1995). Expansion of the poly alanine tract in OPMD mutant PABPN1 enhances its aggregation property. However, no loss of cellular function due to this mutation has been detected (Messaed & Rouleau, 2009). In addition, PABPN1 can associate with RNA polymerase II along the chromatin axis before or shortly after the transcription initiation, and the assembly of PABPN1 on the poly (A) tract may be coupled to transcription (Bear et. al., 2003). Studies have shown that PABPN1 remains associated with the released mRNA-protein complex (mRNP) until it reaches the cytoplasmic side of the nuclear pore. Very little PABPN1 is present in the cytoplasmic side of the nuclear envelope suggesting perhaps during or shortly after passage through the nuclear pore PABPN1 is displaced by PABPC1 (Abu-Baker et. al., 2005; Afonia et. al, 1998; Calado et. al., 2000; Kraus et.al., 1994) . PABPN1 has also been shown to interact with the SKI-binding polypeptide (SKIP) transcription factor and stimulate myogenesis (Kip et. al., 2001). Depletion of PABPN1 in myoblasts prevents myogenesis and reduces the length of the poly (A) tract of mRNAs (Apponi et. al., 2010). Because, of the vital role of PABPN1 in mRNA metabolism it is not certain that whether the observed effect on myogenesis was related to a specific effect on myogenesis or due to impairment of global mRNA metabolism. The poly A extension mutant of PABPN1 appears to function normally in pol(A) tail elongation process. Since PABPN1 can interact with both RNA and polypeptide partners, like other RNA binding proteins additional interacting partners such as micro RNAs and signaling polypeptides may soon be detected to suggest additional cellular functions for PABPN1 .

3. Pathology of OPMD

The most distinctive feature of OPMD is the presence of intranuclear filamentous inclusions in skeletal muscle fibers. The inclusions are composed of aggregates of mutant PABPN1 and several additional proteins which will be discussed later. The filaments are less than 0.25 nm long tubular in structures with an average outer diameter of 8.5 nm and an inner diameter of 3 nm. Approximately 2-5% of nuclei of skeletal muscle cells of OPMD patients show the presence of nuclear inclusions (Tome et. al., 1997). The myo-pathological patterns of OPMD, which progress with age include variations in the diameter of muscle fibers; increase in the number of internal nuclei; and increased presence of endomysical connective tissues. Also, a variable number of typical rimmed vacuoles are found in OPMD muscle fibers (Uyama et. al., 2000). Recently, neuro-pathological abnormalities have also been described in some OPMD patients (Boukriche et. al., 2002). Recent studies using a transgenic mouse model of OPMD severe muscular atrophy of the fast glycolytic muscles were observed. Transcrsiptome analyses of the OPMD mouse muscle showed deregulation of a large

number of genes by expression of OPMD mutant PABPN1 but not by the wild type PABPN1, and approximately one third of the affected genes were associated with muscle atrophy (Trollet et. al., 2010). There is a strong correlation between the presence of intra nuclear inclusions (INI) and the PABPN1 mutation. All patients whose muscle biopsy showed 8.5nm intranuclear filaments have expanded PABPN1 alleles (Bao et. al., 2002). This view was further supported by the formation of large mutant PABPN1 aggregates similar to the INI in cell culture models ectopically expressing human PABPN1. In cell culture models over expression of both wild type and mutant PABPN1 resulted in aggregate formation (Tavanez et. al., 2005). However, the wild type PABPN1 formed aggregates more slowly than what was observed with the poly alanine expanded mutant PABPN1 (Schinchuk et.al., 2005). More apoptotic cell death was also observed in cells with mutant PABPN1 aggregates (Bao et. al., 2002; Fan et. al., 2001; Tavanez et. al., 2005).

4. Misfolded protein aggregates

Misfolding of proteins may lead to formation of protein aggregates. This process could be triggered by many factors including oxidative and temperature stresses. In addition, point mutations and expansion of poly alanine or poly glutamine tracts may increase aggregation by favoring the assembly of the unfolded or partly folded monomers into the early pre-fibrillar species which can turn into aggregates with more distinctive morphologies called protofilaments or protofibrils. The protofibrils may act as seeds where other misfolded polypeptides are recruited to form insoluble fibrillar aggregates (Chiti et. al., 2003). For many years it was believed that the ability to form amyloid fibrils is limited to small number of proteins. However, more recent studies have uncovered that for some proteins the fibrillar aggregates represent a biochemically active form. For examples the aggregated fibers known as curli produced by *E. coli is* important for cell adhesion (Chapman et. al., 2002); yeast prion Sup35, a translation termination factor (eRF3) forms aggregates (Tuite et. al., 2011). Many studies support a role of Aβ amyloid aggregates in sealing capillaries following traumatic injuries (Atwood et. al., 2003). Studies have shown that *Aplysia* cytoplasmic poly adenylation element binding protein (CPEB) exists in two different structural isoforms, one being the soluble isoform and the other as a prion like protein aggregates, and interestingly the CPEB prion is involved in stimulating synaptic growth and long term memory (Si et. al., 2003). It is therefore, conceivable that the poly alanine expansion of PABPN1 results in a gain of function(s). Most RNA binding polypeptides, are capable of participating in a variety of cellular processes, thus it is likely that the OPMD mutation of PABPN1 results in the loss of some cellular functions while gaining one or more new biological activity. Future research needs to be directed towards unraveling additional cellular functions for both mutant and the wild type PABPN1.

Studies using synthetic peptides consisting of varying lengths of the homopolymeric alanines were used to determine the length of the alanines tract that leads to inclusions. Conformational transition to insoluble aggregates was found to depend on the length as well as concentration, temperature, and incubation time. No β sheet complex was detected with less than 8 alanines while ala 10- 15 showed significant conversion of monomeric peptides to β-sheet aggregates. Homopolymers of 15 or more alanines residues showed the highest conversion to aggregates under all conditions examined (Schinchuk et. al., 2005). These results agree with the in vivo observations that the OPMD mutant PABPN1 is more

prone to form aggregates than the wild type PABPN1. *In vitro* studies also showed that fibril formation can be induced by low amounts of both mutant and wild type fibrils serving as seeds. Atomic force microscopy revealed morphlogic differences between wild type and mutant fibrils. In addition, the wild type fibrils were less resistant to solubilization by chaotropic agent guanidinium thiocyanate than what was observed for the mutant fibrils. Examination of the kinetics of fibril formation with PABPN1 fragments containing the polyalanine tract in real time using tryptophan fluorescence suggest that fibril formation coincides with the burial of the tryptophans in the fibrillar core. These studies did not detect any soluble pre-fibrillar intermediates suggesting that the unfolded soluble form directly converts into folded insoluble structure (Schinchuk et. al., 2005).

5. Cellular stress and PABPN1 aggregates

A variety of cellular stresses results in the formation of misfolded proteins, and in order to maintain cell viability and subsequent recovery when physiologically favorable conditions return most organisms produce a family of chaperones known as the heat shock proteins (HSPs) which helps the proper folding process (Daugaard et. al., 2007). It appears that the presence of mutant PABPN1 aggregates but not the wild type cohort in the nuclei produces a modest stress response resulting in the increase of HSP70 expression. Treatment of cells with indomethacin or $ZnSO_4$ augmented the stress response and further induction of HSP70 expression was observed (Figure 2). In addition, expression of HSP27, HSP40 and HSP105 also increased. Both ibuprofen and $ZnSO_4$ treated cells showed reduced level of protein aggregates and apoptotic cell death. Furthermore, in the drug treated cells all four HSPs were colocalized with the PABPN1 (Wang & Bag, 2008). These results suggest that HSPs interact with misfolded PABPN1 and are able to dissociate the aggregates by refolding it into its native form. Similar results were obtained by heat shock treatment of cells and over
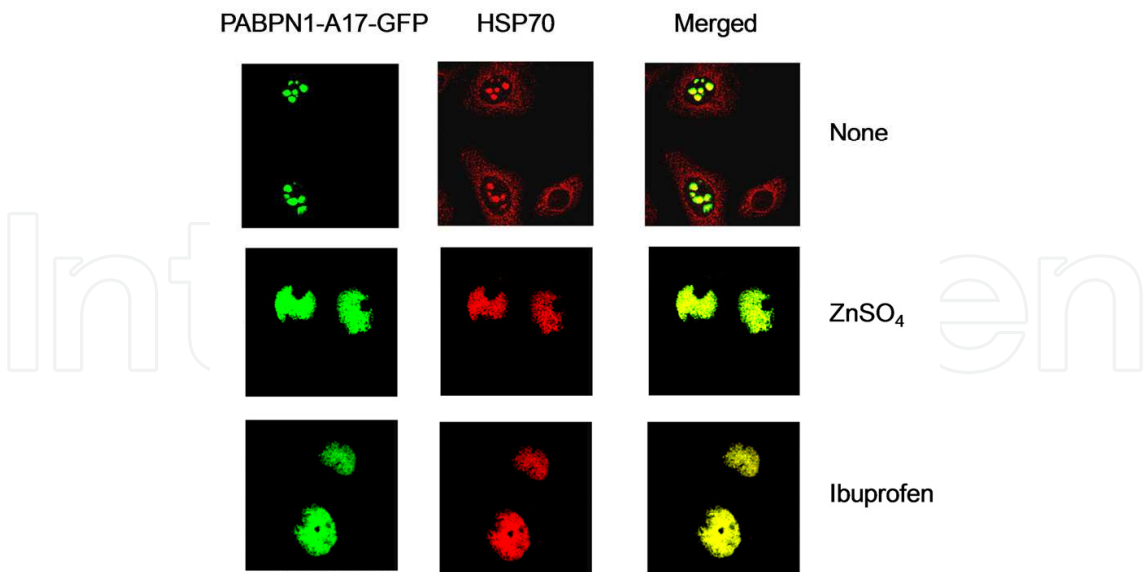

Fig. 2. Effect of different agents on aggregate formation by PABPN1-A17–GFP. HeLa cells were transfected with the PABPN1-A17–GFP expression vector and 48 hours after transfection, cells were treated with the indicated agents for 6 h and following a 24 h recovery period cells were examined for green fluorescence by confocal microscopy. HSP70 was detected by immunofluorescence with Texas red conjugated secondary antibody.

expression of HSP70 alone (Bao et. al., 2002' Wang et. al., 2005; Wang & Bag, 2008). Studies in our laboratory showed that deletion of the ATPase domain of HSP70, which is important for its chaperone function abolishes its ability to dissociate the mutant PABPN1 aggregates (unpublished).

6. Effect of PABPN1 on myogenesis

Despite the essential cellular function of PABPN1 in biogenesis of mRNA the pathologic symptoms are only seen in a restricted group of skeletal muscles such as the extraocular and pharyngeal muscles. Therefore, in addition to its role in mRNA biogenesis PABPN1 may be needed for proper differentiation of myogenic cells, which may be lost in mutant PABPN1 due to expansion of the poly alanine tract. Studies using a myoblast cell culture model showed that over expression of PABPN1 facilitates differentiation of myoblasts into myotubes (Kim et. al., 2001). PABPN1 has been shown to interact with SKIP which share significant homology to several transcriptional co activators such as Bx42 of *Drosophila melanogaster* (Wieland et. al., 1992), and mammalian NcoA-62 (Baudino et. al., 1998). SKIP appears to co-operate with PABPN1 in stimulating E box mediated tarnscription in presence of myoD by forming a hetero trimeric complex (Kim et. al., 2001. The N terminal domain of PABPN1 alone is necessary for interacting with SKIP. The C terminal domain including the RNA binding domains of PABPN1 are dispensable for its role in myogenesis (Kim et.al 2001). Although the poly alanine expanded PABPN1 also binds to SKIP *in vitro* (Tavanez et. al., 2009) it is not clear whether it can cooperate with MyoD to stimulate E box regulated transcription. However, this prospect is conceivable because of the location of poly alanines expansion is within the SKIP binding domain of PABPN1.

In addition to a loss of function in myogenesis the mutant PABPN1 may also gained a function albeit fortuitously, by trapping essential myogenic factors. Studies from our laboratory have indeed supports this hypothesis. We have shown that both myf 5 and Pax 3 co-localize with mutant PABPN1 aggregates but not with the wild type PABPN1 (Figure 3). Ectopic expression of wild type PABPN1 in C2C12 mouse myoblasts had a small beneficial effect on the expression level of various muscle specific proteins including myoD, myogenin, muscle creatine kinase, α-actin and slow troponin C. In contrast, expression of mutant PABPN1 reduced the abundance of those proteins (Figure 4) (Wang & Bag 2006).

The experimental results discussed above may explain why skeletal myogenesis could be affected but very little is known regarding specific targeting of the craniofacial muscles. To address this issue it has been proposed that continuous remodeling of the extraocular myofibers could result in selective loss of this muscle cells (Wirtschafter et. al., 2004). Since *in vivo* myonuclei of most skeletal muscles are post mitotic, therefore, continuous myofiber remodeling in extraocular muscle will require upregulation of genes in cell cycling and renewal of differentiated muscle cells (Wirtschafter et. al., 2004). The negative effect of mutant PABPN1 on myogenesis would show more pronounced effect on muscles that require more frequent rejuvenation than the other skeletal muscles over many years.

7. Protein aggregates and cell death

A direct connection between protein aggregation and cell death is controversial (Andrew et. al., 2000; Fan & Rouleau, 2003; Rubinsztein, 2002). Studies using live cell imaging have
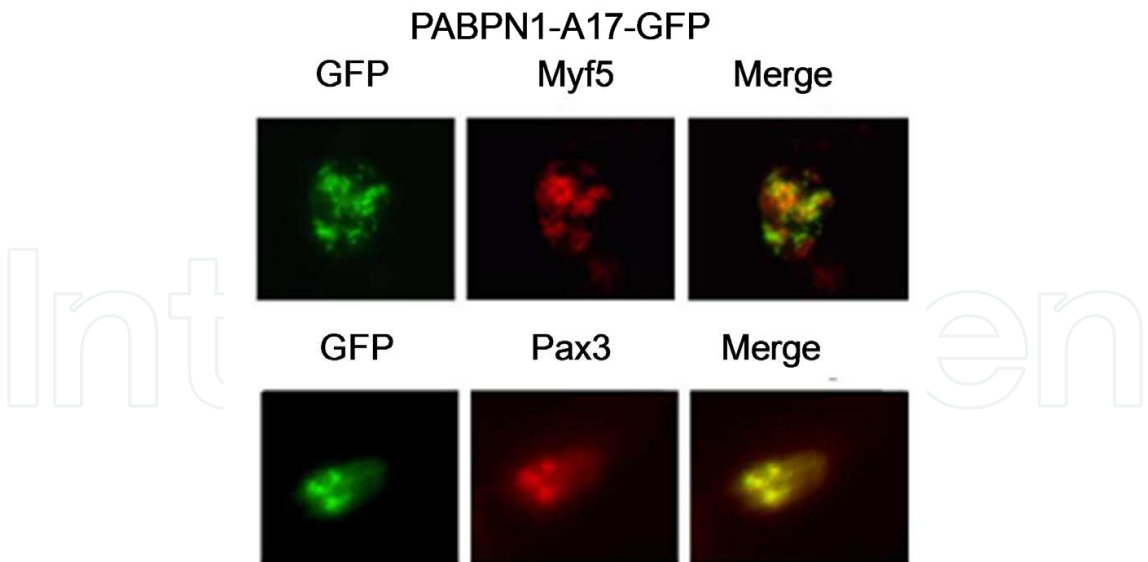
Fig. 3. Co-localization of Pax3/7 and Myf-5 in PABPN1-A17-GFP-transfected cells. Cells grown on coverslips were transfected and 48 h after transfection cells were fixed in methanol and incubated with the appropriate antibody. The green fluorescence of PABPN1-GFP and the red fluorescence of Texas red-conjugated secondary antibody were observed by fluorescence microscopy.
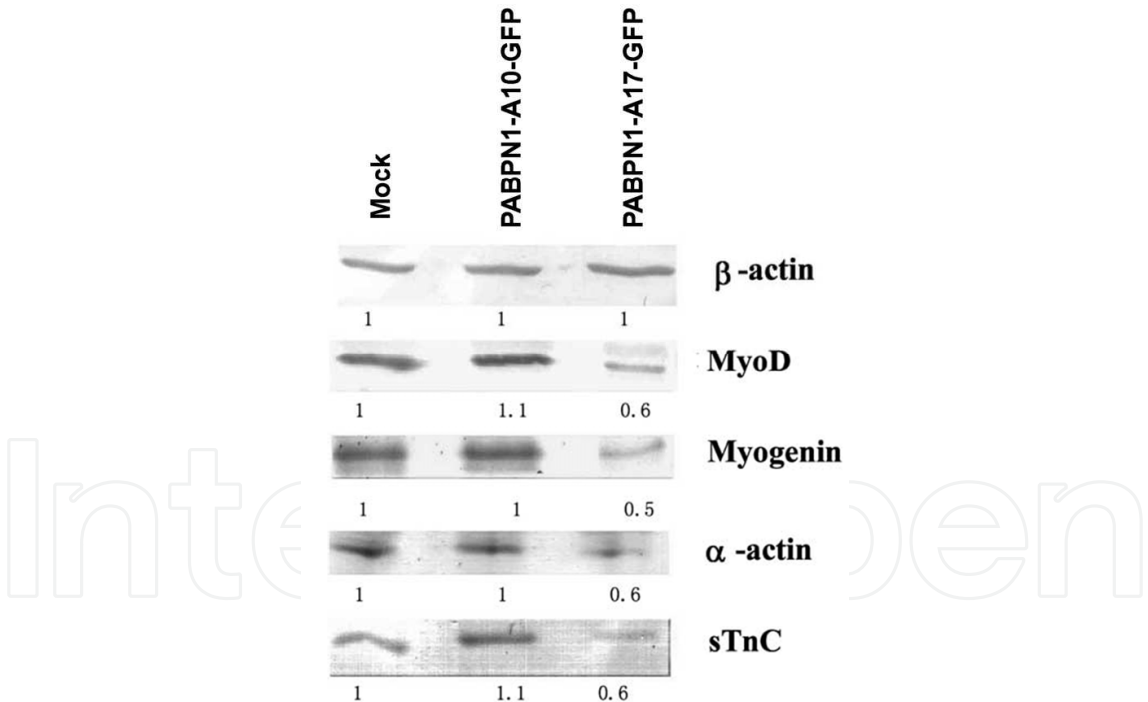

Fig. 4. Expression of muscle-specific proteins in PABPN1-A10 (or A17)-GFP-transfected cells. Cells were transfected with the appropriate Plasmid DNA after 2 days in the 2 days inthe differentiation medium, cells were lysed and the levels of various muscle proteins, were determined by Western blotting using jappropriate antibodies The Western blots were scanned and the levels of muscle proteins in transfected cells were determined and corrected for the difference in loading and transfection efficiency. The polypeptide levels in PABPN1-A17-GFP-transfected cells relative to that of the PABPN1-A10-GFP expressing cells are given at the bottom of each lane.

shown that cells expressing poly glutamine expanded huntingtin survives better than those without aggregates. It is believed that aggregation sequesters this protein and improves cell survival whereas the soluble oligomeric form of mutant huntingtin is more toxic to the cell (Arresate et. al., 2004). Whether the same is true for PABPN1 is not clear. The wild type PABPN1 naturally exists in a functional oligomeric form and is also present as aggregates in the speckles but these are not known to cause cell death. Two overlapping oligomerization domains are found within the C-terminal region of PABPN1. These domains are necessary for oligomerization and aggregation. Therefore, if the oligomeric form of mutant PABPN1 is toxic to the cell it must assume a different structure than that of the wild type protein. Indeed this may be the case since the sub nuclear location of wild type and mutant PABPN1 are different. The wild type PABPN1 was shown by immuno fluorescent microscopy to co-localize with the splicing factor SC35 and the nuclear matrix associated protein PML while the mutant PABPN1 did not (Messaed et. al., 2007; Tavanez et. al., 2005). However, this observation is paradoxical since both proteins seems to function normally in the poly adenylation process, and presence of wild type PABPN1 in the speckles is related to its role in transcription and splicing coupled polyadenylation. In contrast to the pro-apoptotic effect of mutant PABPN1 the wild type PABPN1 demonstrated anti-apoptotic function in mammalian cells. The wild type PABPN1 apparently up regulates the translation of anti apoptotic protein X-linked inhibitor of apoptosis (XIAP) which prevents activation of caspase 3 by inhibiting caspase 9 (Davies & Rubinsztein, 2011). Thus a loss of anti-apoptotic function of mutant PABPN1 may be responsible for cell death in OPMD muscles.

Several studies using cultured non-muscle cells as experimental models showed that strategies that reduced protein misfolding also decreased aggregate formation and cell death. Ectopic expression of the molecular chaperones HSP40 and HSP70 in cells transfected with the mutant PABPN1 reduce aggregate formation and cell death (Abu_Baker et. al., 2003; Bao et. al., 2002; 2004). Also anti-amyloid compounds such as Congo red and doxycyclin can reduce PABPN1 aggregate formation and cell death in a cell culture model (Bao et. al., 2004). We have shown that $ZnSO_4$, 8-hydroxyquinoline, indomethacin and ibuprofen induced HSP 70 expression, and nuclear localization of both HSP70 and the constitutive chaperone HSC 70 in mutant PABPN1 expressing HeLa cells, and reduced the formation of mutant PABPN1 aggregates and cell death (Wang et. al., 2005)

In several chronic neurodegenerative disorders including Alzheimer's, Huntington's, and Parkinson's, caused by the formation of protein aggregates, there is evidence that programmed cell death (apoptosis) may be involved (Desjardins & Ledoux, 1998). Apoptotic cell death has also been observed in cell models and transgenic mouse models of OPMD (Fan & Rouleau, 2003; Hino et. al., 2004; Dion et. al., 2005). However, the molecular mechanisms causing apoptosis remain elusive. Many studies suggest that in the aggregate containing cells, apoptosis proceeds through the up regulation of the tumor suppressor protein p53 (Bae et. al., 2005; Biswas et. al., 2005; Hooper et. al., 2007). Stabilization of p53 within the cell further leads to the activation of down stream proteins like PUMA (p53-upregulated modulator of apoptosis), Bax (Bcl-2-associated X protein) and Bad (Bcl-2-associated death promoter) that change the permeability of mitochondrial and endoplasmic reticulum membranes (Biswas et. al., 2005; Mattson, 2004). These events lead to the release of cytochrome C from mitochondria and calcium from the ER, which further activates the

enzyme called caspase (Mattson, 2004). The cascades of proteolytic activities initiated by caspases are believed to trigger various morphological and biochemical aspects of the cell death process. Furthermore, in Huntington's disease, the GAPDH-Siah1 apoptotic pathway (Hara et. al., 2005) facilitates nuclear translocation of mHtt protein and the resultant neurotoxicity (Bae et. al., 2006). In addition to mitochondrial alterations, ER stress, due to the presence of misfolded polyglutamine has also been linked to the cell death in Huntington's and Alzheimer's disease models (Zhao & Ackerman, 2006).

We have demonstrated that although in OPMD cell death is restricted to a sub class of skeletal muscles, non muscle cells in culture also underwent apoptosis. This was not unexpected since PABPN1 is ubiquitously expressed. We found that in HeLa cells aggregation of the poly alanine expansion mutant PABPN1, favors apoptosis over necrosis or ER stress as cell death pathway. At the molecular level, cascades of biochemical events lead to apoptotic cell death due to the accumulation of mutant PABPN1 aggregates. Our results suggest that the apoptotic response to the accumulation of mutant PABPN1 aggregates was initiated by nuclear translocation of the glycolytic enzyme GAPDH. In the last decade several studies have shown that GAPDH is a multi-functional protein (Chuang et. al., 2005). This enzyme usually resides in the cytoplasm as a tetrameric active enzyme. As a response to cellular stress, the catalytic cysteine 150 of GAPDH is S-nitrosylated by nitric oxide, generated by the induction of inducible nitric oxide synthase (iNOS). It has been shown that Nitrosylated GAPDH binds to Siah1, an E3 ubiquitin ligase, and is transported to the nucleus as an inactive enzyme by piggy backing Siah1 (Hara et. al., 2005). The downstream target of GAPDH in the nucleus is p53 (Sen et. al., 2008). In our study, following ectopic expression of mutant PABPN1, we observed that the abundance of total as well as phosphorylated p53 was increased (Figure 5). p53 a tumor suppressor protein with wide ranging biological function including cell cycle arrest, apoptosis, and its abundance is known to increase in response to a variety of cellular damage (Green & Kroemer, 2009). In cells under stress, post translational modifications, especially phosphorylation and acetylation contribute to p53 stabilization and hence its activation (Sakaguchi et. al., 1998). It has been proposed that phosphorylation of p53 at ser 46 modulate the p53 gene promoter selection thereby dictating the fate of the cell to undergo p53 mediated apoptosis and/or growth arrest (Mayo et. al., 2005). The importance of phosphorylation in p53 mediated apoptosis was further underlined, by demonstrating that mutation of Ser46 to Ala decreases the ability of p53 to induce apoptosis (Oda et. al., 2000). It is known that p53 mediated apoptosis can be carried out by both transcription dependant and independent manner (Chuang et. al., 2005; Pietsch et. al., 2008). We found that in mutant PABPN1 cells, abundance of p53 and its phosphorylated isoform (p-p53) increases (Figure 5). Furthermore we also observed a redistribution of p53 in the nucleus and the mitochondria of mutant PABPN1 transfected cells (Figure 6). There was also a concomitant rise in the p53 transcription targeted pro apoptotic protein: Puma (Figure 5).

Thus, it appears that in mutant PABPN1 cells, activated p53 could be translocated to the nucleus and triggered the transcription dependant apoptosis (Wang et. al., 2007). This might be the reason why we did not observe acetylation of p53, since p53 acetylation occurs predominantly in transcription independent apoptosis (Yamaguchi et. al., 2009). However, both the transcription dependant and independent pathways are not necessarily mutually
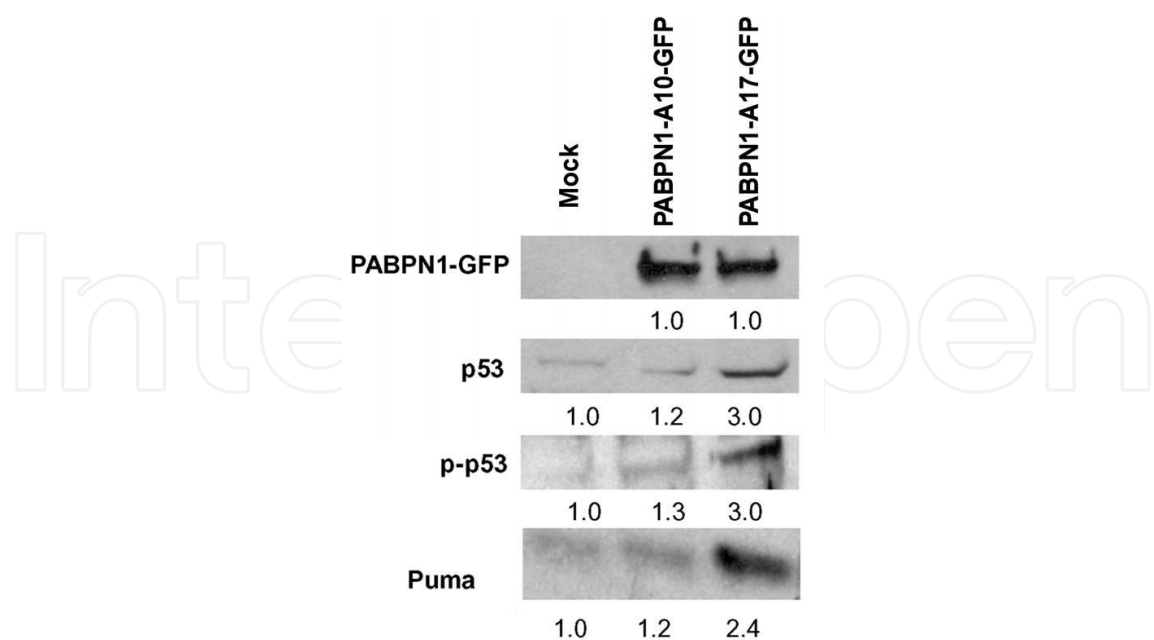
Fig. 5. PABPN1-A17 upregulates p53 and p53 mediated transcription: Following transfection, cells were harvested after 72 hours in SDS loading buffer. Whole cells extracts from PABPN1-A10, 17–GFP and mock-transfected HeLa cells were analyzed for apoptosis related proteins by western blotting. β-actin and GAPDH were used as loading controls.

exclusive. In fact, it has been suggested that the transcription dependent nuclear action of p53 cooperates with its transcription-independent, cytosolic/ mitochondrial action through activation of the *PUMA* gene (Chipuk et. al., 2005). Upon activation, Puma triggers apoptosis by releasing the p53 from its association with Bcl2 to activate Bax (Uo et. al., 2007;Wang et. al., 2007; Zhang et. al., 2009). Puma may also directly interact with Bax, promoting its mitochondrial translocation (Chipuk et. al., 2004; Zhang et. al., 2009). Puma may release p53 from its complex with Bcl2. The released p53 then could oligomerize the monomeric Bax in the cytosol causing the latter to induce mitochondrial outer membrane permeabilization (MOMP) (Dewson et. al., 2003; Jurgensmeier et. al., 1998). The activation of Bax by p53 is known to occur by a 'hit and run' style transient molecular associations (Chipuk et. al., 2004; Moll et. al., 2006; Green & Kroemer, 2009; Pietsch et. al., 2008).

It will be important to examine if a similar apoptotic signal contributes to cell death in muscle cells. In a recent study with the OPMD mouse model over-expression of Bcl2 rescued muscle weakness and apoptosis (Davies & Rubinsztein, 2011), therefore suggesting a similar Bax/Bcl2 pathway for apoptosis in both muscle and non-muscle cells. However, in the OPMD mouse the effect of Bcl2 on muscle weakness was transient, thus other cell death pathways may also contribute to cell death when Bax is inactivated by Bcl2. It is conceivable in the light of our observations in HeLa cells that increase in p53 level might eventually release Bax from Bcl2 mediated inactivation by sequestering Bcl2.

There are several pathways for apoptosis. The precise mechanism of apoptosis depends on developmental programs and the nature of the inducer (Green & Kroemer, 2009; Pietsch et. al., 2008). The Puma/Bax dependent pathway is usually triggered by a variety of cellular

stress such as heat shock and oxygen stress (Uo et. al., 2007; Zhang et. al., 2009). The results of our study suggest that accumulation of misfolded protein aggregates also induces stress related apoptosis. In this context it is interesting to note that as discussed in a previous section a small but reproducible induction of a number of heat shock proteins including HSP70, HSP27, HSP40, and HSP105 was observed in mutant PABPN1 expressing cells (Wang & Bag 2008). Furthermore, all of these HSPs were found to be translocated to the cell nucleus and co-localize with the mutant PABPN1 aggregates. Further induction of HSPs using ibuprofen or indomethacin was shown to reduce the aggregate burden and apoptosis in mutant PABPN1 expressing cells (Wang & Bag 2008). HSP 70 has been shown to prevent heat stress induced apoptosis in cultured cells by preventing Bax translocation without directly interacting with Bax (Stankiewicz et. al., 2005). The mechanism how HSP70 induction with ibuprofen in mutant PABPN1 expressing cells prevents cell death will be of interest for further investigation.

The accumulated evidence supports a biochemical catastrophe model where loss of function combined with adventitious gain of function due to poly alanine expansion leads to cell death. The gain of function includes but not limited to increased aggregate formation, interaction with HSPs, trapping of various transcription factors and mRNAs. In studies using mtHtt aggregate formation in *C. elegans* it was shown that presence of mtHtt aggregates interferes with proper folding of normal cellular proteins and cell death could result from not only the aggregate burden of the mutant protein but also by the misfolding of many normal proteins which results in at the least reduction in the abundance of biologically active important cellular proteins (Gidalevitz et. al 2006). Since most studies measured protein abundance using western blotting techniques which does not measure the level of biological activity of the protein these changes has remained under explored.

The following hypotheses might explain the late onset and specificity of cellular targets *in vivo* of OPMD mutation: I) although aggregates can be cleared through proteasome degradation pathway, this pathway is not sufficient to completely prevent accumulation of aggregates; ii) aging is also associated with collapse of protein homeostasis resulting in accumulation of misfolded normal cellular proteins (Taylor & Dillin, 2011) and when this is combined with a mutation in an aggregate prone protein such as the PABPN1, it greatly increases accumulation of both mutant PABPN1 and many normal nuclear proteins in the intranuclear aggregates; iii) aging may also affect the ability to clear the aggregates through proteasome mediated decay; iv) although both muscle and non muscle cells undergo apoptosis, non-muscle cells are renewed through stem cells, in contrast since myogenesis is affected due to loss of function of mutant PABPN1, regeneration of differentiated muscle cells are affected; iv) skeletal muscles in adults are renewed only when injury occurs but in contrast the adult extraocular muscles undergo continuous remodeling (Wirtschafter et. al., 2004), therefore, extraocular muscles are more susceptible to the loss of myogenic role of mutant PABPN1.

8. Novel therapies for OPMD

Mouse and Drosophila models have been used to develop new therapies to treat OPMD. Administration of anti-amyloid agent doxycyclin to OPMD mice significantly reduced

aggregate formation in muscle cells. In addition to its anti-amyloid properties doxycyclin also acts as an anti apoptotic agent to protect muscle cells (Davies et. al., 2006; 2008). In another study cystamine protected against the cytotoxicity of mutant PABPN1 in the OPMD mouse. Cysatmine inhibits transglutaminase 2 which is elevated in OPMD muscle cells (Davies et. al., 2010). Studies using the Drosophila model of OPMD single chain antibody against PABPN1 also produced nearly complete rescue of OPMD muscles and restored muscle gene expression (Chartier et. al., 2009). In a nematode model of OPMD the inhibitor of Sir2 sitinol also showed promising results in protecting muscle cells from apoptosis (Catoire et. al., 2008). Gene therapy approach using Bcl2 over expression also rescued OPMD mouse from muscle degeneration (Davies & Rubinsztein, 2011).

Several anti amyloid agents such as the disaccharide trehalose, and Congo red also worked in cell culture models of OPMD (Davies et. al., 2006). In our laboratory we have used ibuprofen, indomethacin, 8-hydroxy quinoline and $ZnSO_4$ to induce HSP 70 expression in HeLa cells. All of these agents significantly reduced the aggregate burden and cell death (Wang et.al., 2005). However these compounds have not been tested in an animal model yet. Ibuprofen's effectiveness was tested in a mouse model of Alzheimer disease without success. However, its conjugation with glutathione greatly improved its effectiveness in reducing aggregate formation and cell death in Alzheimer rats (Pinnen et. al., 2010). Zn^+ is an essential mineral nutrient and many people supplement their diet with it, as such, it is potentially a desirable treatment option. Similarly 8-hydroxy quinoline is also an approved agent used in animal feed as antimicrobial and antparasitic agent (Raether & Hanel, 2003). Its effective dose in the cultured cell is within the range of non-toxic dose. Various derivatives of this drug demonstrated their ability in reducing amyloid plaques in clinical trials on Alzheimer patients (Gouras & Beal 2001; Di Vaira et. al., 2004). In addition to the use of various pharmacological approaches in developing new therapies for OPMD, *in situ* myoblasts transfer by local administrations (Mouly et. al., 2006) or localized gene therapy of affected muscles using Bcl2 or HSP 70 gene expression should be considered.

9. References

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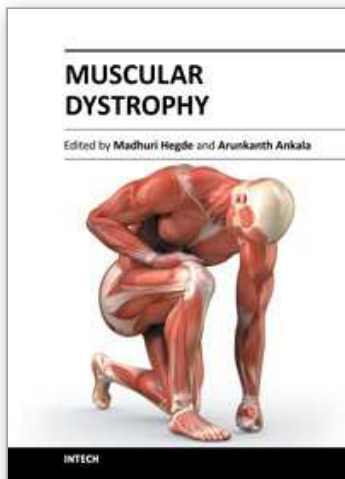
Muscular Dystrophy

Edited by Dr. Madhuri Hegde

With more than 30 different types and subtypes known and many more yet to be classified and characterized, muscular dystrophy is a highly heterogeneous group of inherited neuromuscular disorders. This book provides a comprehensive overview of the various types of muscular dystrophies, genes associated with each subtype, disease diagnosis, management as well as available treatment options. Though each different type and subtype of muscular dystrophy is associated with a different causative gene, the majority of them have overlapping clinical presentations, making molecular diagnosis inevitable for both disease diagnosis as well as patient management. This book discusses the currently available diagnostic approaches that have revolutionized clinical research. Pathophysiology of the different muscular dystrophies, multifaceted functions of the involved genes as well as efforts towards diagnosis and effective patient management, are also discussed. Adding value to the book are the included reports on ongoing studies that show a promise for future therapeutic strategies.

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