Pur α and Hsp70 as Protective Agents Against RNA-mediated Toxicity in Fragile X-Associated Tremor/Ataxia Syndrome

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Fragile X-associated tremor/ataxia syndrome (FXTAS) is a rare neurodegenerative disorder that is considered a pre-mutation of full Fragile X syndrome. It is characterized by 55-200 rCGG repeats in the fragile X retardation 1 gene (FMR1) mRNA that mediate toxicity leading to symptoms of ataxia, action tremor, parkinsonism, and cognitive decline. Pur α, a protein known to aid in replication and transcription of DNA, binds to these repeats as a Repeat Binding Protein and has been discovered to mediate FXTAS neurodegeneration. Similarly, heat shock protein Hsp70 has also played a role in suppressing neurodegeneration. The molecular mechanism of pathogenesis and its relationship with FMR1 mRNA, Pur alpha, and Hsp70 is still debated. We hypothesize that Pur α and Hsp70 mediate a protective response to RNA-mediated toxicity in FXTAS. To test this hypothesis, we aim to 1) validate rCGG-mediated toxicity in vivo, 2) manipulate the expression of Pur α and Hsp70 in vivo, and 3) investigate the location of binding sites on Pur α and rCGG repeats through in vitro and cell culture studies. This research will provide critical evidence for possible treatments and provide the scientific community with a greater understanding of this rare disorder whose mechanisms are still largely unknown.

Background

Fragile X-associated disorders make up the most common cause of inherited mental retardation (1). In these disorders, trinucleotide repeat expansions (>200 CGG repeats) of the fragile X mental retardation 1 gene (FMR1) lead to cognitive and emotional disabilities. Normally, individuals have less than 55 CGG repeats within their FMR1 gene. A subset of these Fragile X-associated disorders, called Fragile X-associated Tremor/Ataxia Syndrome, is an adult onset neurodegenerative disorder with an FMR1 allele premutation: an expansion of 50 to 200 CGG repeats.

The average age of onset for Fragile X-associated tremor/ataxia syndrome (FXTAS) is 50 years. However, death is usually caused by secondary complications associated with aspiration pneumonia or infection. FXTAS is an X-linked dominant disorder and only requires one allele copy to increase the risk of developing the disease. It is more common in males, with a prevalence of approximately 1 in 8,000 (1). Females are less susceptible to inheriting the disorder because they have two X chromosomes instead of only one X chromosome, which males have. The disorder starts with the development of movement disorders such as intention tremors and after several years of onset, gait ataxia (2). Other features of the disease include parkinsonism, autonomic dysfunction, and cognitive decline (3).The pathology of FXTAS is characterized by the formation of ubiquitin-positive intranuclear inclusions throughout the brain in both neurons and astrocytes (4). In addition, a decrease in Purkinje cells inside the cerebellum can be seen, with intranuclear inclusions forming in a few dentate neurons and cerebellar astrocytes (4). Previous researchers propose that the molecular pathogenesis is linked to FMR1 mRNA gain-of-function toxicity (5,6). In premutation carriers (55-200 CGG repeats), FMR1-encoded protein (FMRP) remains near normal levels, while levels of FMR1 mRNA is elevated 8-fold (7,8). FMRP usually functions as an aid in healthy cognitive development, female reproductive function, synaptic protein synthesis, and mRNA translation (9-10). Unlike other neurodegenerative diseases that mainly have a build up of aggregated proteins in the intranuclear inclusions, previous researchers found that FXTAS patients also contained FMR1 mRNA in the intranuclear inclusions, making this disorder unique (9). However, there are also various proteins found in FXTAS-associated inclusions. Pur α and Hsp70 are two of these proteins that might have an importance in mediating rCGG repeat toxicity (12-14). Pur α normally aids the replication and transcription of DNA into RNA and is labeled as a repeat binding protein (RBP). One study by Jin et al. (2007) suggests that Pur α may be sequestered in FXTAS, leading to the inhibition of its normal function. One possible mechanism of dysfunction suggests that Pur α excessively binds to the repeats and is limited in its ability to fulfill other functions in the cell (13). It has also been shown that overexpression of Pur α has been linked with decreased neurodegeneration in FXTAS flies. Hsp70 is a heat shock chaperone protein that protects against cell stress by preventing protein misfolding and aggregation. Studies have found nuclear accumulation of Hsp70 transcript in FXTAS fly models (9). These nuclear accumulations could lead to deficits in the nuclear export of mRNA and lead to FXTAS neurodegeneration. Overexpression of Hsp70 has also been linked with decreased neurodegeneration in FXTAS flies (14). Both Hsp70 and Pur α seem to have some sort of role in FXTAS pathogenesis as they are found in the intranuclear inclusions. However, more evidence is needed to determine whether the main culprit of FXTAS toxicity is due to the mRNA or the translated protein. It is also unclear how Pur α and Hsp70 together might influence mRNA toxicity. We hypothesize that Pur α and Hsp70 mediate a protective response to RNA-mediated toxicity in FXTAS. Findings from this research will stimulate new therapeutic routes to combat FXTAS and provide information on the toxic molecular mechanisms involved with rCGG repeats.

Relevance

Broader Relevance

FXTAS is a rare disorder that is estimated to affect 1 in 8,000 men over the age of 55. However, the primary mechanism of pathogenesis is still debated. Although it is a rare disorder, research into FXTAS treatments and pathogenesis can provide insight into more prevalent repeat expansion diseases, such as Fragile X Syndrome, the most inherited cause of intellectual disabilities in the world. Patients with FXTAS have symptoms that include cognitive decline, intention tremors, gait ataxia, parkinsonism, and more. Through this research, treatments, therapeutic strategies, and the cause of the disease could be uncovered to understand and treat patients suffering from FXTAS symptoms.

Intellectual Merit

By examining the rCGG repeats of FMR1, we will gain additional information on the functionality and conservation of the gene. In addition, research into FXTAS has only been briefly examined in cell lines, so it will be interesting to discover the effects of the mutated mRNA and manipulation of Pur α/Hsp70. Furthermore, we will also obtain insight into both in vitro and in vivo models to assist future research in related or unrelated fields.

Specific Aims

The aim of our proposal is to find evidence of FMR1 mRNA-induced toxicity in human cell cultures and transgenic mice, to study the interaction between varying CGG repeats in FMR1 mRNA through underexpression or overexpression of Pur α and HSP70 in human cell cultures and transgenic mice, and to find the binding sites on Pur α and FMR1 mRNA in vitro. We hypothesize that Pur α and HSP70 mediate a protective response to RNA mediated toxicity in FXTAS. These in vivo and in vitro studies will provide insight into FXTAS pathogenesis and possible targets for therapy.

1. *Demonstrate FMR1 mRNA- mediated toxicity in FXTAS:*

We will first create SK cell lines and transgenic mice with varying CGG repeat levels of the FMR1 mRNA. Vectors that carry a stop codon upstream the varying CGG repeats will be utilized. We will evaluate cell survival, inclusion formation, and motor function of our model organisms with and without FMR1 mRNA translation.

2. *Evaluate the manipulation of Pur α and Hsp70 levels in vivo and in vitro:*

In SK cell lines and transgenic mice with varying numbers of rCGG repeats, we will use RNA interference to knock down Pur α and Hsp70 levels. We will also overexpress Pur α and Hsp70 levels by using vectors carrying multiple copies of the PURA and HSPA1A gene. We will evaluate cell survival, inclusion formation, and motor function.

3. *Locate binding site of Pur α and FMR1 mRNA repeats:*

We will truncate Pur α domains and mutate the FMR1 gene to create varying forms of FMR1 mRNA. Truncated Pur α will be allowed to interact with full length FMR1 mRNA using a streptavidin gel-shift assay.

Wild-type Pur α will be allowed to interact with mutated FMR1 mRNA using RNA immunoprecipitation. This will allow us to identify Pur α and mRNA binding regions.

Research Methods and Design

1. *Demonstrate FMR1 mRNA- mediated toxicity in FXTAS:*
Rationale

The molecular pathogenesis of FXTAS is still unclear. Varying lines of evidence link pathogenesis to an RNA-mediated gain-of-function toxicity. In FXTAS carriers, levels of FMR1 mRNA are elevated while levels of FMRP stay at relatively normal levels (7,8). There is also evidence that reduced translational efficiency of FMR1 mRNA in FXTAS cells leads to reduced FMRP, which is important for normal cognitive development (15). FMR1 mRNA was also found in inclusions of FXTAS patients (12). One possible mechanism of dysfunction is the production of multiple toxic species from the translation of the CGG repeats in the FMR1 mRNA. In order to test whether the mRNA or the formation of toxic species is more harmful, we will evaluate mutations that block the translation of the CGG repeats of FMR1 mRNA in cell lines and transgenic mice. We hypothesize that FXTAS is linked more with RNA toxicity and that blocking translation of FMR1 mRNA CGG repeats will lead to little change in cell survival, inclusion formation, and motor function.

1. Creation and evaluation of SK cell lines and knock-in mice with varying FMR1 CGG repeats:

Genomic DNA will be selected from males carrying 40 (control), 62, 99, and 180 length FMR1 CGG repeats (16). Southern blot hybridization and sequencing will verify the CGG repeat lengths. The varying CGG repeat FMR1 genes will be cloned into the pcep4 vector, a vector modified to express eGFP while under the control of a TRE promoter (17). The resulting plasmid will then be transfected into human neuroblastoma-derived SK-N-MC cells (SK). (17-18). After plating the SK cells, doxycycline will be used to induce the TRE promoter to express the FMR1 gene with varying CGG repeats. Fluorescence microscopy will be performed to check for stable expression of the vector and Quantitative real-time polymerase chain reaction (qRT-PCR) will evaluate the quantity of mRNA made. Transfected SK cells will be compared to non-transfected SK cells, which will serve as the negative control. Three assays will be utilized to measure cell toxicity. In the MTT tetrazolium colorimetric assay, cells will be incubated with MTT to a concentration of 0.5mg/ml for 2 hours. A spectrophotometer will be used to measure absorbance to quantify viable cells (19). Cell survival will be assessed by using Trypan blue to selectively color dead cells blue in order to count the clear, surviving cells. Immunohistochemistry will be used to observe formation of intranuclear inclusions by immunostaining for αB-crystallin, ubiquitin, and staining nuclei with DAPI (9). Next, we will make mouse lines with varying FMR1 CGG repeats. We will replicate the methodology used by Jackson et al. (2009) to create knock-in mice with 40 (control), 62, 99, and 180 length FMR1 CGG repeats (20). A qRT-PCR will be run to confirm FMR1 mRNA expression. These mice will be compared to the negative control WT mice. We will assess the pathology of the knock-in mice by using immunohistochemistry to observe inclusion formation. Immunostaining will use antibodies specific for αB-crystallin and ubiquitin (9). Furthermore, mice will perform a rotarod test and the beam-walking assay to observe locomotion. Statistical test one-way ANOVA will be run for each of these analyses to evaluate significant differences between the varying rCGG repeat groups.

Prediction: When comparing the conditions to each other, we predict that increasing the number of CGG repeat mRNAs will lead to reduced cell viability, found by the Trypan blue and MTT tetrazolium colorimetric assay. It will also lead to increased formation of intranuclear inclusions, found by immunohistochemistry in cell lines and transgenic mice. There will be disruption of movement ability in the transgenic mice. If this prediction is correct, it would provide evidence that the mRNA leads to toxicity. However, if the prediction is incorrect, then it would be hypothesized that toxicity is not mediated by mRNA and could be attributed to the translation process.

2. Generation and evaluation of human SK cell lines with blocked FMR1 mRNA translation:

SK cell lines will be created to have FMR1 mRNA with varying CGG lengths but translation will be blocked. Vectors containing the varying CGG repeats will undergo site-directed mutagenesis (Stratagene) in order to introduce a stop codon upstream the CGG repeats of the FMR1 gene, according to the protocol used by Todd et al, 2013 (10). The resulting plasmid will be transfected into SK cells. Western blot will be performed to show no production of protein by using the FMRP antibody (rAM1) (21). Cell survival will be evaluated using the Trypan blue and MTT tetrazolium colorimetric assay. Immunohistochemistry will be used to observe formation of intranuclear inclusions by immunostaining for αB-crystallin and ubiquitin. These results will be statistically compared to the quantifications found in cell lines that still translated mRNA. Knock-in mice will be created with blocked mRNA translation. The vector containing a stop codon upstream the varying CGG repeats will be incorporated into the mouse genome by replicating the procedure by Hall et al. 2010 (22). Immunohistochemistry will find inclusions by using antibodies for αB-crystallin and ubiquitin (9). Mice will perform a rotarod test and the beam-walking assay to observe changes in locomotion. Results will be statistically compared to quantifications found using mice with translated mRNA. Statistical test one-way ANOVA will be run for each of the analyses.

Prediction: We predict that cell toxicity is mainly a product of mRNA-mediated toxicity. Blockage of translation will lead to little change in cell viability and inclusion formation when compared to the quantifications found from part 1 of aim 1 for cell lines and knock-in mice. There will be similar motor impairment in the mice as well. Significant increases in cell viability, decreased inclusions, and decreased motor impairment could mean that toxicity is mainly due to the toxic proteins made from translation of varying CGG repeat FMR1 mRNA.

2. *Evaluate the manipulation of Pur α and Hsp70 levels in vivo and in vitro:*

Rationale: Pur α is known to have an important function in the replication and transcription of DNA while Hsp70 is essential for protection against cell stress and protein misfolding. Evidence shows that Pur α binds to rCGG repeats, lowering the neurodegeneration associated with FXTAS (13). The RNA helicase Rm62 in Drosophila, which directly interacts with Pur α, was found to regulate Hsp70 mRNA transport outside of the nucleus in order to be translated into protein (14). Similar to Pur α, over-expression of Hsp70 was associated with suppression of rCGG repeat toxicity, leading to cell survival that was quantified in Drosophila eyes. It is possible that Pur α and Hsp70 have therapeutic possibilities to modulate rCGG toxicity and FXTAS symptoms, although it is still unclear how Pur α and Hsp70 mediate mRNA toxicity. Over-expressing and under-expressing both Pur α and Hsp70 will give future researchers a better understanding of the FXTAS mechanism of dysfunction. We hypothesize that over-expression of these proteins will reduce rCGG repeat mediated cell death, inclusion formation, and motor dysfunction.

1. Creation of SK cell line and transgenic mice with Pur α and Hsp70 knock down

Genomic DNA from individuals carrying 40 (control), 62, 99, and 180 length FMR1 CGG repeats will undergo PCR amplification (16). These genes will be cloned into the integrating vector pLEM8 (Hirst 1998) in order to have stable, ongoing expression of the genes when placed in the SK cells. The resulting plasmid will be transfected into SK cells and integrated into the chromosome by homologous recombination (24). Gene placement will be verified by southern blot and sequencing analysis. These SK cell lines will now have DNA with varying numbers of CGG repeats. SK cells already carry the HSPA1A and PURA genes that code for Hsp70 and Pur α protein. RNA interference will be used to degrade mRNA in order to inactivate Pur α and Hsp70 expression by following the protocol in Salvio et al. (2015) (25). Western blots will then be performed to show no production of Hsp70 and Pur α protein by using anti-iHSP70 and anti-Pur α. Cell survival will be evaluated using the Trypan blue and MTT tetrazolium colorimetric assay. Immunohistochemistry will be used to observe formation of intranuclear inclusions by immunostaining for αB-crystallin and ubiquitin. These transfected SK cell lines will be compared to non-transfected SK cell lines. By following the Hall et al. (2010) protocol, we will create knock-in mice by integrating vectors for varying CGG repeats (22). RNA interference will be used again to inactivate Pur α and Hsp70 expression by following the protocol in McCaffrey et al. (2002) (26). Immunohistochemistry will find inclusions by using antibodies for

αB-crystallin and ubiquitin (9). Mice will perform a rotarod test and the beam-walking assay to observe changes in locomotion. These knock-in mice will be compared to WT mice. Lastly, we will run the statistical test one-way ANOVA to evaluate significant differences between the transfected SK cell lines and the non-transfected SK cell lines.

Prediction: We predict that knock-down of Pur α and Hsp70 in SK cells will lead to decreased cell survival and an increase in the number of inclusions when compared to the SK cells from aim 1, step 1. Knockdown in mice will lead to more inclusions and increased motor dysfunction when compared to mice from aim 1, step 1. If the pathogenesis is similar or better in knock down mice or SK cells, it would give evidence that Pur α and Hsp70 might not play a role in preventing pathogenesis in FXTAS.

2.Creation of FXTAS SK cell lines and knock-in mice with overexpressed Pur α and Hsp70 levels

The therapeutic possibility of Pur α and Hsp70 will be assessed by overexpressing Pur α and Hsp70 levels in the SK cells. In order to overexpress the proteins, we will subclone multiple copies of the PURA gene into the pPB-N-His vector and multiple copies of the HSPA1A gene into the pEGFP-C3 vector. These plasmids will be transfected into SK cells with varying numbers of CGG repeats (27). Ni-Affinity Chromatography will be used to show expression of the PURA gene and Fluorescence Microscopy will be performed to identify expression of HSPA1A. In order to evaluate the levels of protein made, western blotting will be performed with monoclonal anti-iHSP70 and anti-Pur α. Trypan blue, MTT tetrazolium colorimetric assay, and immunohistochemistry with anti-αB-crystallin and anti-ubiquitin will be used to assess cell survival and inclusion formation. These transfected SK cell lines will be compared to non-transfected SK cell lines with varying CGG repeats from aim 1, step 1. Knock-in mice will be created using vectors with multiple PURA genes and HSPA1A genes after homologous recombination in ES cells (22). Immunohistochemistry will find inclusions by using antibodies for αB-crystallin and ubiquitin (9). Mice will perform a rotarod and beam-walking test to observe changes in locomotion. These knock-in mice will be compared to WT mice and mice with varying CGG repeats created in aim 1, step 1. Statistical test one-way ANOVA will be run for each of the analyses.

Prediction: We predict that the Western blot assay will show increased levels of Pur α and HSP70 protein because multiple copies of the gene will be placed into the vectors. Since Pur α and Hsp70 are associated with suppression of rCGG repeat toxicity (14), we predict there will be increased cell survival, less inclusion formation, and better motor function. If there is the same level of cell survival, inclusion formation, and motor function, this suggests that Pur α and Hsp70 does not mediate rCGG repeat toxicity.

3. *Locate binding site of Pur α and FMR1 mRNA repeats:*
Rationale:

The rCGG repeats of the FMR1 mRNA are linked with FXTAS pathogenesis in patients (7,8). There must be between 55 and 200 CGG repeats in an individual's DNA sequence in order to be affected by FXTAS. FMR1 genes with more than 200 CGG repeats are linked with the full mutation (FXS). As stated prior, Pur α is an important protein that is specific to FXTAS. It binds to these CGG repeats and is located within the intranuclear inclusions of FXTAS patients (13). Identification of Pur α as a RBP has been a significant discovery but the exact location of binding to the FMR1 mRNA repeats has not been investigated and can be considered critical information to further understand the pathogenesis of FXTAS. Possible therapeutic agents could be developed to target the identified Pur α binding sites. In order to find the binding sites, Pur α domains will be truncated and FMR1 genes will be mutated to create varying forms of FMR1 mRNA. In vitro interactions between the protein and mRNA will reveal the binding site for both the mRNA and protein. We hypothesize that Pur α binds between the 55-200 repeats because Pur α protein binding is seen specifically in FXTAS. We also hypothesize that Pur α will bind to the mRNA through one of its repeat domains.

1. Locate the mRNA binding site on the Pur α protein in vitro

Pur α has five domains; an N terminal Glycine-rich domain (1-41), three repeat domains (42-106, 120-182, 197-252), and a C terminus (246-322) (28). The PURA gene will be subcloned into the pPB-N-His vector and PCR will be performed with designed primers to create truncations of PURA over its domains. The PCR products will be verified by agarose gel electrophoresis (29). These vectors containing truncations will be transfected into SK cells and the proteins will then be purified as described by Giasson et al., 2001 (30). Biotinylated-FMR1 mRNA with 200 CGG repeats will be incubated each time with a different truncated Pur α protein in vitro (23). The binding reaction will be run on a polyacrylamide gel and Streptavidin beads will be used to capture the rCGG-protein complexes. A biotinylated-RNA probe will be used to show whether there is binding between the rCGG mRNA and the different truncations of the protein.

Prediction: We predict to see varying sizes of PCR product bands on the agarose gel due to the truncated PURA gene. As there is an increase in truncation of Pur α domains, there will eventually be no band, showing RNA-protein complexes forming with a certain truncation. This will allow for the identification of the domain where Pur α binds to the FMR1 mRNA. We predict it will be in one of the three repeat domains (42-106, 120-182, 197-252) of Pur α.

2. Locate the Pur α binding site on the FMR1 mRNA in vitro

Genomic DNA from an individual carrying 300 length FMR1 CGG repeats will undergo Polymerase Chain Reaction (PCR) amplification (7,8). PCR with designed primers will be used to create mutated FMR1 genes. These mutated genes will be cloned into episomal vector pcep4 and the resulting plasmid will be transfected into SK cells. RT-PCR will be used to evaluate levels of FMR1 mRNA expression for each mutation. The cells will be lysed and RNA-protein complexes will be immunoprecipitated using anti-Pur α (31). The RNA-protein complexes will be separated by RNA extraction and the RNA will be identified by reverse transcription into cDNA for sequencing (31). In addition, we will create Pur α expressing GST and incubate it with the mutated mRNA. GST has a high affinity for GSH-coated beads and the resulting complexes will be pulled down on a gel. We will extract the RNA to identify the binding sites (32).

Prediction: We predict that Pur α binds to the FMR1 mRNA with 55-200 CGG repeats. Mutated FMR1 gene will produce varying sizes of PCR product bands. Through sequencing, the mRNA sequences with no mutation and formed protein-RNA complexes would give evidence to where the Pur α may be binding. If no significant sequences are found, Crispr will be used to make deletions on the FMR1 gene to produce different sizes of mRNA. Overall, both of these methods would lead to the determination of the FMR1 mRNA main binding sites for Pur α.

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