The molecular genetics of premutation and full mutation alleles of the *FMR1* gene

Rea Erika Biacsi

Dr. Karen Usdin, PhD
Supervisor
Section Chief
Section on Gene Structure and Disease
Laboratory of Molecular and Cellular Biology
National Institute of Digestive, Diabetes and Kidney Diseases
National Institutes of Health

Prof. Dr. László Orosz
Program director
Classical and Molecular Genetics Program

Prof. Dr. Anna Erdei
Director of Doctoral School
Biology Doctoral School

Faculty of Sciences
Eötvös Lóránd University

1. Introduction

Fragile X syndrome

The Fragile X syndrome is the most common X-linked inherited mental retardation, its prevalence is 1:4000 in boys and 1:8000 in girls. It caused by a CGG•CCG triplet repeat expansion to ≥200 ('full mutation') in the 5' untranslated region of the Fragile X Mental Retardation 1 gene (*FMR1*), which leads to silencing of the gene and a lack of FMRP. Since the sequence of the promoter and open reading frame of these alleles is unchanged, the potential exists to ameliorate the symptoms of FXS by reversing the gene silencing.

How repeats of this length cause silencing is unknown, but the silencing process involves DNA methylation as well as epigenetic changes. The methylation can have direct and indirect effects; it can directly inhibits the binding of methylation sensitive transcription factors, and indirectly induces chromatin condensation which blocks transcription initiation and elongation.

Silencing and reactivation of the FMR1 gene

The silenced gene is associated with overall H3 and H4 hypoacetylation. Lysine 4 and 9 of histone H3 are the only 2 specific modifiable sites that have been examined thus far. In individuals with FXS, the levels of histone H3 acetylated at K9 (H3K9Ac) and H3 dimethylated at K4 (H3K4Me2) are decreased relative to the normal gene, while the level of H3K9 dimethylation (H3K9Me2) is increased. By analogy with other genes that have been studied more extensively, we would expect that there are a number of other histone residues that are differentially methylated or acetylated when the FMR1 gene is aberrantly silenced. In previous studies, treatment of FXS cells with histone deacetylases inhibitors had little or no effect on FMR1 gene reactivation and the drugs were very toxic. The DNA methyltransferase inhibitor 5-aza-2-deoxycytidine (5azadC) produced significant

gene reactivation but is also extremely toxic and requires DNA replication to be effective.

Premutation carriers

Individuals with repeat numbers between 55 and 200 are called 'premutation carriers' and they are at risk for Fragile X-associated tremor/ataxia syndrome (FXTAS). Females in this repeat-range often have primary ovarian insufficiency (FXPOI) and their children are at risk for inheriting full mutation alleles. In premutation carriers the *FMR1* mRNA level in blood is 2 to 10-fold higher than in unaffected individuals, while the FMRP level decreases with increasing repeat number. The symptoms of FXTAS and FXPOI are thought to arise from the toxic effects of large numbers of CGG-repeats in the *FMR1* mRNA from premutation alleles. However, there are some symptoms seen in premutation carriers that are similar to those seen in full mutation carriers. These symptoms may arise from a problem with translation of transcripts with long CGG-repeat tracts.

Mice models

An *FMR1* knockout (KO) mouse model for Fragile X syndrome is available to study the effects of the loss of FMRP. However, since these animals dos not contain an expanded CGG•CCG-repeat tract, they cannot be used to study repeat-induced phenomena such as germline expansion, epigenetic changes, and translation difficulties. These phenomena can be studied only in mice that carry a premutation or full mutation allele. The first premutation mice model generated showed no consistent effects on *FMR1* mRNA levels or FMRP. Furthermore this model did not show the big expansions which are characteristic for the premutation patients. However, Dr Usdin's laboratory has generated a second premutation mouse model that does seem to better recapitulate some of the features of human premutation carriers.

I have participated as a collaborator in studies on these premutation animals. I have described the work done by me in the results section of my thesis, and mentioned with work done by my collaborators in the discussion section.

2. Aims of the study

1. Recently it has become apparent that not only do some HDACs act preferentially on specific lysines on different histones, but they also target certain genes for deacetylation. Thus the previous studies did not role out a role of HDACs in gene silencing in FXS. Previously tested HDAC inhibitors only targeted the members of the histone deacetylase class I, II and IV. Therefore this work set out to examine members of the Class III histone deacetylases and their role in *FMR1* gene silencing.

2. In order to help understand the effect of these drugs and to help more effective gene reactivation strategies, we also studied the epigenetic changes associated with the silencing and reactivation of the *FMR1* gene.

3. In the *Fmr1* knock-in mice model, generated in our lab by my colleagues, I examined the relationship between repeat number and FMRP expression.

4. In collaboration with a colleague, we examined the intergenerational changes in the CGG•CCG-repeat number in these animals.

3. Materials and Methods

1. We used histone deacetylase and DNA methyltransferase inhibitors on FXS lymphoblastoid and fibroblast cells to study the mechanism of *FMR1* gene silencing.

2. To follow the changes in *FMR1* mRNA expression, we used quantitative real-time PCR. To monitor the FMRP levels we used western blotting and to investigate the methylation status of the alleles we carried out a thermal denaturation analysis of PCR amplified, bisulfite converted DNA.

3. We followed the epigenetic changes by chromatin immunoprecipitation (ChIP).

4. We transfected normal and FXS fibroblast cells with normal and dominant negative SIRT1 expression vectors to investigate the SIRT1 role in the silencing process. We detected the expression level by quantitative real-time PCR and we investigated the SIRT1 binding to the normal and full mutation alleles by ChIP.

5. We used a dominant negative human MOF (hMOF) expression vector to investigate the role of hMOF in *FMR1* gene reactivation. After the transfection of fragile X fibroblasts with the dominant-negative expression construct, we examined the *FMR1* expression changes by quantitative real-time PCR.

6. To genotype the premutation carrier mice we used PCR on tail DNA using primers flanking the repeat.

7. We used a radioactive PCR technique to amplify the premutation alleles and high resolution sequencing gels to monitor the size of the premutation alleles.

8. We investigated the effect of increasing repeat number on FMRP levels by Western Blotting.

4. Results

1. Class III histone deacetylase inhibitors like splitomicin, were able to reactivate the silenced *FMR1* gene leaving the DNA methylation status unchanged.

2. The Class III HDAC, SIRT1, is enriched on full mutation alleles, and blocking SIRT1 deacetylation, eliminated the effect of splitomicin.

3.Blocking the activity of the histone acetylase, hMOF, which is necessary for H4K16 acetylation, also greatly reduced the effect of splitomicin.

4. Treatment of FXS with 5-azadC also resulted in H4K16 acetylation.

5. The premutation mice, previously generated in our lab, show an increase of the *Fmr1* mRNA level and decrease of the FMRP level with increasing repeat number, like human premutation carriers.

6. The premutation allele is extremely unstable in the mouse germline. However most of these changes were very small and showed a paternal expansion bias. In this respect this germline instability resembles what is seen on transmission of small premutation alleles in humans.

7. Big expansions were observed in the KI mice model, but only at a low frequency, even with repeat numbers that show ~100% probability of expansion to the full mutation range when transmitted in humans. Furthermore, unlike what is seen in humans, paternal alleles also showed these big expansions.

5. Conclusions

1. Unlike the previously studied histone deacetylases inhibitors, which were not able to reactivate the silenced *FMR1* gene significantly, splitomicin was able to reactivate the silenced *FMR1* gene and it did so without causing DNA demethylation. Thus in postmitotic cells, like neurons, such compounds may be more useful than DNA methylation inhibitors in the reactivation of the *FMR1* gene.

2. SIRT1, a member of the Class III histone deacetylases, plays an important role in the silencing of the *FMR1* gene, while hMOF is involved in reversing the gene silencing. These two proteins are thus potential targets for new therapeutic strategies to reactivate the silenced gene.

3. During the reactivation of the *FMR1* gene, the H4K16 residue becomes acetylated, while the DNA methylation status is unchanged. Taken together with fact that 5-azadC treatment also increased H4K16 acetylation, it suggests that H4K16 deacetylation is a downstream consequence of DNA methylation and a late event in the silencing process. This information may help develop new pharmacological strategies for *FMR1* gene reactivation.

4. The decrease of the FMRP level with increasing repeat number observed in the brains of Fragile X premutation mice resembles what is seen in the blood of human carriers of premutation alleles. Since some premutation carriers show symptoms reminiscent of those resulting from an FMRP deficiency, it may well be that premutation carriers suffer not only from the consequences of RNA toxicity, but also from the effect of the FMRP deficiency as well. This has ramifications for the effective treatment of some premutation carrier symptoms.

5. The expansion frequency is high in the premutation mice model but most of the expansions are small even when alleles are in the >120 repeat range. In addition, large expansions are seen on paternal transmission. These phenomena differ from

what was seen in humans. It is possible that the threshold for expansion differs in mice and humans and that in mice there is no selection against large expansions in germline cells. Despite the differences between mice and humans with respect to these large expansions, this is the first mouse model of the FX premutation in which large expansions are seen at all. Using this model to better understand these differences may help us unravel the mechanism responsible.

6. The increase in the *FMR1* mRNA levels and the decrease in FMRP with the increasing repeat number are also similar to what are seen in humans. Thus these mice may provide a good model system to study the effect of the premutation on brain and ovarian function.

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