Understanding the molecular basis of fragile X syndrome

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Fragile X syndrome, a common form of inherited mental retardation, is mainly caused by massive expansion of CGG triplet repeats located in the 5′-untranslated region of the fragile X mental retardation-1 (FMR1) gene. In patients with fragile X syndrome, the expanded CGG triplet repeats are hypermethylated and the expression of the FMR1 gene is repressed, which leads to the absence of FMR1 protein (FMRP) and subsequent mental retardation. FMRP is an RNA-binding protein that shuttles between the nucleus and cytoplasm. This protein has been implicated in protein translation as it is found associated with polyribosomes and the rough endoplasmic reticulum. We discuss here the recent progress made towards understanding the molecular mechanism of CGG repeat expansion and physiological function(s) of FMRP. These studies will not only help to illuminate the molecular basis of the general class of human diseases with trinucleotide repeat expansion but also provide an avenue to understand aspects of human cognition and intelligence.

INTRODUCTION

Fragile X syndrome is one of the most common forms of inherited mental retardation with the estimated incidence of 1 in 4000 males and 1 in 8000 females (1,2). The syndrome is transmitted as an X-linked dominant trait and with reduced penetrance (80% in males and 30% in females) (1,2). Fragile X syndrome is associated with a fragile site, designated FRAXA (Fragile site, X chromosome, A site), at Xq27.3 near the end of the long arm. The clinical presentations of fragile X syndrome include mild to severe mental retardation, with IQ between 20 and 60, mildly abnormal facial features of a prominent jaw and large ears, mainly in males, and macroorchidism in postpubescent males. Many patients also display subtle connective tissue abnormalities, hyperactive and attention deficit disorder and autistic-like behavior (1,2). In 1991, the molecular basis of fragile X syndrome was revealed by positioning cloning and the 4.4 kb (7,8). Within the 4.4 kb of FMR1 transcript, a CGG trinucleotide repeat is located at the 5′-untranslated region (5′-UTR). Among normal individuals, this CGG repeat is highly polymorphic in length and content, often punctuated by AGG interruptions (9–13). The normal repeat size ranges from 7 to ~60, with 30 repeats found on the most common allele. In most affected individuals, CGG repeats are massively expanded over 230 repeats (full mutation) and becomes abnormally hypermethylated, which results in the silence of the FMR1 gene. Alleles with between 60 and 230 CGG repeats are called premutation. They are generally unmethylated with normal transcript and protein level, but are extremely unstable during transmission to next generation (1,14). Expansion of premutation into full mutation can only occur by maternal transmission and depends on the length of the maternal premutation. Due to X-linkage, affected males have more severe phenotypes than affected females, whose phenotype is modulated by the activation ratio of the normal X chromosome. Identification of other mutations of the FMR1 gene, such as deletions and point mutation among patients with usual phenotype but without fragile site expression, firmly established that the FMR1 gene is the only gene involved in the pathogenesis of fragile X syndrome (15,16). Thus, the absence of the FMR1 gene product, fragile X mental retardation protein (FMRP), is the typical cause of fragile X syndrome. The FMR1 gene is widely expressed in both human and murine tissues (17). Multiple FMRP isoforms, through extensive alternative splicing at the 3′ end, appear in a variety of tissues, and some of them are present at different quantitative levels (7,18,19). FMRP has been implicated in RNA binding and is possibly involved in translational control (20,21). An animal model of fragile X syndrome was created using gene targeting (22). The FMR1 knockout mice exhibit macroorchidism and a subtle deficit in learning and memory, which mimic the human phenotype.

Recent research on fragile X syndrome has been focused on two areas. (i) Instability and methylation of CGG repeats when and how does CGG repeat instability occur? What is the molecular mechanism of DNA methylation of expanded CGG repeats? (ii) Physiological function(s) of FMRP: what genes are the targets of FMRP in vivo? As an RNA-binding protein,
what role(s) does FMRP play in translation? How does the absence of FMRP lead to deficit in learning and memory during development? Here we will review the latest progress made in these two areas towards understanding the molecular basis of fragile X syndrome.

**INSTABILITY AND METHYLATION OF CGG REPEATS**

In the normal population, the CGG repeat is polymorphic but inherited in a stable fashion. The repeat is cryptic with interspersed AGG repeats most often found at repeat positions 10 and 20 (11). The CGG repeat length variation is found at the 3’ end of the repeat and it appears that AGG triplets play a crucial role in the maintenance of repeat stability (11). Most premutation alleles have either no AGG or only a single AGG interruption at the 5’ end of the repeat (10,13,23). Loss of these interruptions leads to alleles with longer perfect CGG repeat tracts, which are prone to expansion into premutation, especially when tracts contain >30 perfect CGG repeats. In contrast to the normal allele, premutation is unstable and may expand to a full mutation or a different sized premutation (6). No new mutations have been reported with all abnormal alleles ultimately derived from premutations, at least in the most recent generations. The risk of expansion of a premutation to a full mutation in the next generation exponentially increases with the number of repeats between 65 and 100. This phenomenon resolves anticipation in fragile X syndrome (also known as Sherman Paradox), which manifests clinically as an increase in the number and proportion of mentally retarded individuals in successive generations in fragile X families (24,25). Remarkably, premutation only expands to a full mutation when it is transmitted by female carrier, but not male carrier. The reason for this is that full mutation males have only premutation-size alleles in their sperm (see below) (26). In the full mutation, the CGG repeat is massively expanded and hypermethylated, which results in the repression of the FMR1 gene and leads to the loss of FMRP. However, mosaicism of both CGG repeat length and methylation status exists in fragile X patients (1). In most affected individuals, somatic variation both CGG repeat length and methylation status exists in fragile gene and leads to the loss of FMRP. However, mosaicism of both CGG repeat length and methylation status exists in fragile X families (28). The supportive evidence is that full mutation carriers are found to be somatic mosaicsisms of CGG repeats (28,29). However, the length of CGG repeats on a particular allele in a differentiated cell derived from these carriers is generally mitotically stable although recent studies show that fully expanded FMR1 CGG repeats exhibit a length- and differentiation-dependent instability in cell hybrids (30,31). Analysis of CGG repeats in gametes of fetuses with full mutation concluded that the testes of a 13 week full-mutation fetus showed no evidence of premutations, whereas a 17 week full-mutation fetus exhibited some germ cells with attributes of premutations (32). Also only full-expansion alleles can be detected in the oocytes of full-mutation fetuses (32). These results indicate that CGG repeat instability happens either during premeiotic or meiotic division, or post-zygotically, but at an extremely early developmental stage prior to germline segregation, which happens on day 5 of development (33). However, the simplest explanation, supported by modeling, is that the full mutation is transmitted in the oocyte and reductional instability accounts for the mosaicism. This awaits direct experimental confirmation and it remains possible that events in the zygote promote expansion.

The molecular mechanism of CGG repeat expansion is still elusive so far. The replication-based model, in which slippage of perfect repeat Okazaki fragments leads to repeat expansion, has been most favored in the literature, at least for premutation to premutation variation. According to this model, for most normal alleles, CGG repeats are interrupted by two AGGs and the long perfect repeat number is <30, which is no longer than the normal mammalian Okazaki fragment length of 25–300 nucleotides; the long perfect repeat can be anchored by AGG interruptions. When the nascent strand contains >30 perfect repeats without an interruption as reference, the Okazaki fragment with CGG repeats only, will tend to slip during the replication, probably also influenced by unusual DNA structure assumed by the repeat (11,34,35). However, this model cannot easily explain how slippage could generate the huge expansion during the transmission from premutation to full mutation. Recently the RAD27 endonuclease in the yeast, or FEN-1, the homolog in mammals, has been proposed to play a key role in repeat instability (36). This endonuclease activity is responsible for the removal of the 5’ ribonucleotide, derived from the RNA primer and the 5’ end of an Okazaki fragment that is displaced by the growing 3’ end of the preceding Okazaki fragment. Deletion of RAD27 in yeast was found to destabilize dinucleotide arrays, favoring repeat addition and also the accumulation of duplication mutations, which indicate that RAD27 might therefore be involved in triplet repeat expansion (37–39). The mammalian RAD27 homolog, FEN-1, functions both in the processing Okazaki fragments during lagging-strand synthesis and in the processing of branched structures which arise in long-patch base excision repair, which indicates that FEN-1 substrate must be single-stranded (40). Structural analysis suggested that triplet repeats could form an intramolecular secondary structure (hairpin), which will be resistant to FEN-1 endonuclease activity (36,41,42). In the absence of RAD27 in yeast, the observation that triplet repeats, including CGG, CAG and CTG, had much elevated frequency of expansions supports this idea (43,44). Also, it was demonstrated that in yeast, secondary structure can indeed inhibit flap processing at triplet repeats in a length-dependent manner by concealing
the 5′ end of the flap that is necessary for both binding and cleavage by FEN-1 (45). So understanding the physiological significance of FEN-1 will be important to solve the mystery surrounding the triplet repeat expansion. In addition, DNA damage has been proposed to play a significant role in triplet repeat expansion. In Escherichia coli, the deficiency of some nucleotide excision repair functions can dramatically affect the stability of long repeat insertions in plasmids (46). A single abasic site analog at the 5′ end of the template of the triplet repeat tract can induce massive triplet repeat expansion during DNA synthesis in vitro (47). Indeed, DNA cleavage of the repeat, due to repair, may initiate strand invasion triggering expansion through an unscheduled DNA replication mechanism, such as gene conversion-type events.

To fully understand the timing and molecular mechanism of CGG repeat expansion, the animal models, which display CGG repeat instability during germline transmission, will be essential. Only in animal models will it become possible to easily study gametogenesis and early embryogenesis at specific time points. Unfortunately, no such animal model is currently available. Creation of animal models with full-length CGG repeats is currently technically challenging due to the extreme instability of full mutation in E. coli. Transgenic mice with FMR1 premutation allele were generated by two separate groups; however, no instability of the CGG repeat was observed in both cases (48,49). Recently, YAC transgenic mice have been generated that carry the human FMR1 gene containing various sized CGG repeats and no instability of CGG repeat was detected (A.M. Peier and D.L. Nelson, submitted for publication). In these transgenic mice, the integration sites of either transgene or YAC are random, which may indicate that triplet repeat instability requires the cis-acting element(s). This is further supported by association studies, although this remains controversial (50). It will be interesting to study the instability of CGG repeats with premutation size at murine FMR1 or adjacent locus using a gene-targeting approach (knock-in). Furthermore, these knock-in mice will be tested in different genetic backgrounds and applied to mutagenesis to define the pathways that lead to triplet repeat instability. However, it is possible that there may exist interspecies differences between human and mouse in this regard and that humans lack mechanisms, present in mice, which stabilize the repeats.

The timing and molecular mechanism of methylation on expanded CGG repeat, which lead to FMR1 transcriptional silencing, are also under extensive study. As discussed earlier, all alleles from intact ovaries of full-mutation fetuses are fully expanded, but unmethylated. However, chorionic villi samples taken at different stages of development were shown to be methylated to various degrees (32). Also, the studies on human lymphoblastoids of full mutation showed that the methylation of individual CpG cytosine is strikingly variable in hypermethylated epigenotype obtained from a single individual (51). These results indicate that the methylation of expanded CGG repeats occurs within a broad window and is a dynamic process. Three distinct families of DNA methyltransferase genes, named Dnmt1, Dnmt2 and Dnmt3, have been identified in mammalian cells, but which DNA methyltransferase(s) is involved in the methylation of expanded CGG repeats is still unknown (52). Recently it has been shown that methylation of CGG repeats causes transcriptional silencing through histone deacetylation (53). In the cells from normal individuals, the 5′ end of FMR1, which contains CGG repeats, is associated with acetylated histones H3 and H4, but acetylation is reduced in cells from hypermethylated full mutation (53). Histone deacetylation at the FMR1 locus, induced by the hypermethylation of expanded CGG repeats, will alter the chromatin structure of the 5′ end of the FMR1 gene, which will lead to transcriptional silencing (Fig. 1). Treatment of fragile X cells with 5-aza-deoxycytidine (5-aza-dC), which induces DNA demethylation through the inhibition of DNA methyltransferase, resulted in reassociation of acetylated histone H3 and H4 with FMR1 and transcriptional reactivation (53,54). But treatment of fragile X cells with different inhibitors of histone deacytlases resulted in little reactivation of the FMR1 gene. However, combining these histone deacetylase inhibitors with 5-aza-dC can lead to greater increase of FMR1 transcripts than 5-aza-dC alone, which suggests a synergistic effect of histone hyperacetylation and DNA demethylation in the reactivation of FMR1 full mutation (55). This reactivation strategy may provide a potential therapeutic approach for fragile X syndrome. However, the side effect of these drugs and the difficulty in efficiently translating FMR1 transcripts with large repeats may limit this approach (27,56).

**PHYSIOLOGICAL FUNCTION(S) OF FMRP**

Since fragile X syndrome is a single gene disorder caused by the absence of FMRP, the question becomes how the absence of FMRP induces the clinical phenotype. The answer to this question requires a full understanding of the physiological function(s) of FMRP. The FMR1 gene is widely expressed in both human and murine tissues. In situ hybridization with the adult mouse tissues showed abundant expression in brain, testes, ovary, esophageal epithelium, thymus, eye and spleen, with moderate expression in colon, uterus, thyroid and liver, and no expression in the heart, aorta or muscle (17). Analysis of the amino acid sequence of FMRP revealed the presence of two types of RNA-binding motif, two nucleic acid protein K homology domains (KH domains) and clusters of arginine and glycine residues (RGG boxes), which suggested that FMRP is an RNA-binding protein (20,21). In addition, a patient carrying a nonsense mutation was identified with unusually severe mental retardation (15). The point mutation alters an isoleucine residue to asparagine at position 304 (I304N), which is located at the second KH domain of FMRP, and this isoleucine is well conserved in almost every KH domain of different proteins (57,58). This further suggested that the RNA-binding property of FMRP is critical for its functions. Also two autosomal homologs of the FMR1 gene, FXR1 and FXR2, have been identified and the overall structures of the corresponding proteins are very similar to that of FMRP (59,60). It has been proposed that due to their similarities, FXR1P and FXR2P can compensate for the function(s) of FMRP in its absence, which leads to both lethal but very mild phenotype in both human and mouse. However, cells from fragile X patients and the FMR1 knockout mice lacking FMRP expression have a normal expression level of both FXR1P and FXR2P (61). The functions of this FMRP family, including FMRP, FXR1P and FXR2P, have been extensively studied in the past several years (62).

Studies of the RNA-binding specificity of FMRP showed that FMRP preferentially binds to poly(G) and poly(U) instead...
Moreover, FMRP can only bind to selective brain mRNAs in vitro, including the 3′-UTRs of myelin basic protein (MBP) and the FMR1 message itself. However, the in vivo binding specificity and the native RNA targets of FMRP remain to be determined. This will be very critical for understanding the physiological functions of FMRP. The other side of this story is which region(s) of FMRP interact(s) with RNA. Due to the presence of KH domains and RGG box within FMRP, it would be assumed that these regions should be responsible for RNA interaction. However, by creating deletion mutants of FMRP, the N-terminal stretch, two KH regions and C-terminal stretch, it has been shown that only the first KH domain but not the second interacts with RNA (64). This result is consistent with the observation that FMRP with a point mutation (I304N) at the second KH domain can bind to RNA, although this mutation can disrupt the structure of the KH domain (63). More interestingly, both the N- and C-terminal regions show RNA-binding ability. Within the N-terminal stretch, no known RNA-binding motif is identifiable and this region binds to poly(G) preferentially, similar to the first KH domains. The RNA binding by the C-terminus, which contains an RGG box, appears non-specific, as it recognizes the bases with comparable affinity (64). Based on these data, it has been proposed that FMRP is a protein with multiple sites of interaction with RNA: sequence specificity is most likely achieved by the whole block that compromises the first ~400 residues, whereas the C-terminus provides a non-specific binding surface (64). The crystal structure of full-length FMRP will ultimately address this question.

FMRP has also been shown to associate with actively translating polyribosomes in an RNA-dependent manner via messenger ribonucleoprotein (mRNP) particles (65,66). First, FMRP–ribosome association is sensitive to low levels of RNase, which removes the mRNA linking translating polyribosomes without disturbing ribosome assembly (65–67). Secondly, EDTA treatment, which dissociates ribosomes into subunits, releases FMRP into complexes with a similar size range to a large mRNP particle, which is ~660 kDa (67,68). However, in the cells with the I304N mutant FMRP, despite normal expression and cytoplasmic RNA association, the
mRNP particles are of smaller size, and do not associate with actively translating polyribosomes (68). Thirdly, nearly all the cytoplasmic FMRP can be co-captured with poly(A) RNA (65,68). Taken together, these data suggest that FMRP associates with ribosomes as a component of mRNP particles. Considering the severe phenotype of patients with point mutations, the association of FMRP with polyribosomes must be functionally important. Recent studies show that FMRP can form dimers, and point-mutation proteins fail to do so, which suggests that dimerization may be critical for the association of FMRP with polyribosomes (D.M. Absher and S.T. Warren, manuscript in preparation). The proteins that make up the FMRP-containing mRNP remain largely unknown. Recently, a cell culture system expressing epitope-tagged FMRP has been developed. By immunoprecipitation, at least six other proteins are shown to form the mRNP particles along with FMRP (69). Two of these proteins are FXR1P and FXR2P, which is consistent with previous findings (60). Additionally, nucleolin, a known component of other mRNPs, is also present in the FMRP-containing mRNP particles (69,70). Identification of the remaining associated proteins will be important for the analysis of FMRP function(s). The observation that FMRP is associated with polyribosomes suggests that FMRP may modulate mRNA translation and influence mRNA instability in the cytoplasm. Indeed, recent studies have shown that FMRP can suppress translation of bound messages in an in vitro translation assay (Z. Li and Y. Feng, manuscript in preparation).

Although FMRP is predominantly localized in the cytoplasm, both a functional nuclear localization signal (NLS) and a nuclear export signal (NES) have been identified within FMRP using FMRP deletion constructs (67). Whereas NLS is located in the N-terminus of FMRP, the NES of FMRP, encoded by exon 14, closely resembles the NES motifs described for HIV-1 Rev and protein kinase inhibitor (PKI) and is sufficient to direct nuclear export of a microinjected protein conjugate (67). The presence of these localization signals suggests that FMRP may shuttle between the nucleus and cytoplasm. Immunogold electron microscopy demonstrated that FMRP exists in the nucleus and has been observed in transit through the nuclear pore (71). Based on these observations, it has been proposed that FMRP plays a role in specific mRNA export from nucleus to cytoplasm (67). Nascent FMRP enters the nucleus and there assembles into an mRNP, interacting with specific RNA transcripts and other proteins. The mRNP particles containing FMRP and its target RNA will be transported to the cytoplasm. Alternatively a possible role in modulating the localization, stability and/or translation of its target mRNA has also been hypothesized. Recently, FMRP has been shown to be a phosphoprotein and a substrate of the Fes non-receptor tyrosine kinase (72). Interestingly, tyrosine phosphorylated FMRP is chiefly located in the nucleus. The significance of phosphorylation status of FMRP in the shuttle between nucleus and cytoplasm is unclear at present, but suggests regulation by signal transduction. The two homologs of FMRP, FXR1P and FXR2P, also contain an NLS and an NES signal motif at a similar position to that seen in FMRP. Although FXR2P has a similar signal motif to FMRP, it was shown that FXR2P and FMRP shuttle between cytoplasm and nucleoplasm, whereas FXR1P shuttles between cytoplasm and nucleolus, which suggests that they may transport different RNAs or have different physiological functions (73). This idea is further supported by the identification of a novel RNA-binding nuclear protein (NUFIP) that interacts with FMRP. NUFIP only interacts with FMRP and not FXR1P and FXR2P (70). It is therefore possible that these three homologous proteins interact with different proteins/sites within the nucleus and may have specific rather than overlapping function(s) there but appear to coalesce, at least partially, in the cytoplasm, forming a common mRNP particle.

Since the primary phenotype of fragile X syndrome, mental retardation, occurs in the CNS, the question becomes what the function of FMRP is in CNS, or how the absence of FMRP leads to cognition deficit. Based on the above findings, it was hypothesized that in neurons FMRP may play a role in the transportation of its target mRNAs from nucleus to cytoplasm, modulate the localization of these mRNAs, and further regulate the local protein synthesis by being part of polyribosomes, which will be important for normal neuronal development and functions (Fig. 2). Several observations support this idea. First, immunogold studies showed that FMRP was localized in neuronal nucleoplasm and within the nuclear pore, which suggests that FMRP indeed participates in the nuclear export of mRNA in the neuron (71). Secondly, ultrastructural studies in rat brain revealed high levels of FMRP immunoreactivity in neuronal perikarya, where it is concentrated in regions rich in ribosomes, in particular near or between rough endoplasmic reticulum cisternae (71). Moreover, FMRP was also observed in large- and small-caliber dendrites, in dendritic branch points, at the origins of spine necks and in spine heads, all known locations of neuronal polyribosomes (71). Thirdly, FMRP can be co-fractionated with synaptosomal ribosomes, which indicates that FMRP may regulate the local protein synthesis of synaptosomes (71). The regulation of protein synthesis within the dendritic spine is important for synaptic development and brain plasticity (74–77). Consistent with this idea, abnormal dendritic spines were observed both in brains of fragile X patients and in FMR1 knockout mice (78,79). This suggests that in most fragile X patients, due to the absence of FMRP, the protein synthesis is misregulated during synapse development, which may lead to mental retardation. In the patient with a point mutation (I304N), FMRP was unable to dimerize and FMRP-containing mRNP failed to associate with the polyribosome, which may sequester FMRP-bound mRNA from translation, thus resulting in an unusually severe phenotype (68) (Fig. 2). The regulation of synaptic-activity-dependent protein synthesis by FMRP is further supported by the observation that the translation of FMRP was increased in rat synaptoneurosomes after stimulation by a specific mGluR agonist (80). To further understand the neuronal functions of FMRP, the animal model will be important. However, currently the only available animal model is the FMR1 knockout mouse, whose behavioral abnormalities are extremely subtle, limiting widespread analysis (81; A.M. Peier and D.L. Nelson, submitted for publication). Development of new animal models including mice with regulatable FMRP expression will be very helpful to dissect the role(s) of FMRP during neuronal development and synapse formation.

**CONCLUSION**

A great deal has been learned about fragile X syndrome since the discovery in 1991 of the FMR1 gene and the molecular
models that display similar CGG repeat instability during transmission through generations will be critical to the understanding of the molecular mechanism of CGG repeat expansion, which remains very elusive. These studies will not only help us to understand the fragile X syndrome but also be important in understanding the molecular basis of at least another 12 human disorders with similar dynamic mutation, trinucleotide repeat expansion. On the other hand, the physiological function(s) of FMRP is being extensively studied to address how the absence of FMRP leads to the clinical phenotype of fragile X syndrome. To ultimately answer this question and develop effective intervention to fragile X syndrome, both molecular genetic and neurobiological approaches have to be combined to bring closer molecular abnormalities to the neurobehavioral phenotypes. New animal models and paradigms of behavior testing have to be developed. These studies will also provide us with insight into the mechanisms of cognition, memory and behavior in human. Moreover, discovery of those genes whose mRNAs interact with FMRP will provide candidate genes for other disorders limiting cognition development.

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