Advances in understanding the molecular basis of FXTAS

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Fragile X-associated tremor/ataxia syndrome (FXTAS) is an adult-onset neurodegenerative disorder among carriers of premutation expansions (55–200 CGG repeats) of the fragile X mental retardation 1 (\textit{FMR1}) gene. The clinical features of FXTAS, as well as other forms of clinical involvement in carriers without FXTAS, are thought to arise from a toxic gain of function of transcriptionally active \textit{FMR1} containing expanded CGG repeats. Although the precise mechanisms involved in rCGG toxicity are unknown, here we discuss the latest advances and models that contribute to the understanding of the molecular basis of FXTAS, and the emerging view of FXTAS as the end-stage of a process that begins in early development.

INTRODUCTION

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disorder that affects many older adult carriers of expanded CGG-repeat alleles in the premutation range (55–200 CGG repeats;\textsuperscript{1}) in the 5\textsuperscript{′} non-coding region of the fragile X mental retardation 1 (\textit{FMR1}) gene (reviewed in \textsuperscript{2–8}). It is estimated that approximately one in 3000 men and a smaller number of women in the general population will develop FXTAS at some point during their lives (\textsuperscript{9–11}), making FXTAS one of the most common single-gene, late-onset neurodegenerative disorders. Larger CGG-repeat expansions (>200 CGG repeats; full mutation) are predominantly transcriptionally silent, resulting in the absence, or highly diminished levels of the \textit{FMR1} mRNA and protein (\textit{FMRP}) (\textsuperscript{12,13}), which plays an important role in synaptogenesis and synaptic plasticity (\textsuperscript{14}). Absence of the protein leads to fragile X syndrome, a neurodevelopmental disorder and the leading monogenic form of cognitive-impairment and autism (\textsuperscript{10,15,16}).

The principal clinical features of FXTAS are progressive intention tremor and gait ataxia, although motor involvement may be more widespread, with aspects of parkinsonism (\textsuperscript{7,17}). Associated features include cognitive decline, often progressing to dementia and frequently including prominent disturbance of executive function; peripheral neuropathy; dysautonomia; and particularly in women, evidence of autoimmune dysfunction (\textsuperscript{4,8,9,18}). The severity of both clinical and neuropathological phenotypes is correlated with the extent of the CGG-repeat expansion within the premutation range (\textsuperscript{18–24}).

Magnetic resonance imaging has established an association with FXTAS of global loss of brain volume with attendant enlargement of ventricular volume, mild-to-severe white matter disease and, in particular, high-signal (T2-weighted) lesions in the middle cerebellar peduncles (\textsuperscript{17,19,25–27}). These gross changes are accompanied by the loss of axons and myelin, significant subcortical astroglial activation and cerebellar Purkinje cell loss (\textsuperscript{21,28}). Moreover, immunocytochemical staining of post-mortem brain tissue from FXTAS cases has revealed the presence of solitary, ubiquitin-positive intranuclear inclusions (Fig. 1) in both neurons and astrocytes in most brain regions, with the numbers of inclusions strongly correlating with the size of the CGG repeat (\textsuperscript{21,28}). More than two-dozen proteins (and the \textit{FMR1} mRNA itself;\textsuperscript{29}) have been identified within the inclusions, based on studies in human, mouse and fly models (\textsuperscript{5,30–36}). Inclusions of similar structure, although with differing compositions, are found in several other neurodegenerative diseases, particularly in the CAG/polyglutamine disorders (reviewed elsewhere in this issue). Inclusions are also found in tissues outside of the CNS in subjects with FXTAS (e.g. testicles and peripheral nerve ganglia) (\textsuperscript{37,38}) and in murine models of the premutation (\textsuperscript{5,36}). Premutation mouse models have also demonstrated that inclusions increase in both number and size with

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The distribution of inclusions in the mouse brain is broadly similar to the distribution in humans and, importantly (see below), are found in Bergmann glia in the cerebellum (34).

The discovery of FXTAS, as well as primary ovarian insufficiency (FXPOI; 11,13,40–42), which are both limited to the premutation range, suggested that an entirely distinct molecular mechanism was operating in the premutation range. In stark contrast to the absence of mRNA and FMRP for hypermethylated, full mutation alleles, carriers of premutation alleles have markedly increased (≏2–8-fold) production of the expanded CGG-repeat mRNA, despite normal or only slightly lowered FMRP levels (43–48). The restriction of the FXTAS clinical phenotype to the premutation range, where the gene is active, coupled with multiple studies of the adverse consequences of expressing the CGG repeat in diverse human, animal and cell models, has given rise to the concept of a ‘toxic’ gain-of-function of the CGG-repeat-containing RNA (1,30,33,35,39,49–54). In this regard, the direct participation of mRNA toxicity may also be important for the CAG-repeat (polyQ) disorders, which are still largely regarded as due to the altered properties of the expanded polyglutamine-containing proteins (reviewed elsewhere in this issue). Using the fly model of spinocerebellar ataxia type 3 (SCA3), Li et al. (55) compared the phenotypes of identical polyQ expansions in ataxin-3, but with changes that disrupt the CAG-repeat element while preserving amino acid identity. Specifically, the CAG-repeat RNA was altered to include an interrupted CAACAG repeat within the polyQ-encoding region. This alteration, with the polyQ sequence, dramatically reduced toxicity; in a separate experiment, expression of an untranslated CAG repeat of pathogenic length did induce neuronal degeneration.

The preponderance of evidence, both clinical and molecular, argues for an RNA-based mechanism leading to FXTAS; however, the precise form of the CGG-repeat RNA responsible for FXTAS pathogenesis is not yet resolved. While there is evidence that the CGG-repeat-containing RNA per se is responsible for the cellular dysregulation, the restriction of clinical involvement to the premutation range, where the gene is active, and much of the animal and cellular work, all formally point to the necessity of transcriptional activity rather than to a specific form of the RNA (Fig. 2).

**AN RNA BASIS FOR FXTAS PATHOGENESIS**

The observation that FXTAS is largely restricted to carriers of premutation alleles suggested that the *FMR1* gene must be transcriptionally active for disease formation (1,28,56), a conclusion drawn earlier in the context of POI (57). The contemporaneous observation of elevated *FMR1* mRNA levels in premutation carriers (43,45,48), coupled with the RNA gain-of-function model proposed for myotonic dystrophy (DM), strongly supported an RNA-based initiation of the cellular dysregulation that underlies FXTAS. For DM, an expa-
Figure 2. Models of transcription-dependent toxicity in FXTAS. RNA toxicity can be direct, as in sequestration of proteins from other functions in the cell (e.g. DM model) and/or activation of proteins upon binding to RNA, which then signal downstream events. RNA involvement may also be indirect, as part of a co-transcriptional mechanism. For example, it is possible that the expanded CGG repeat promotes the formation of R-loops which in turn lead to DNA damage (e.g. of the displaced strand) and a consequent DNA damage response involving γH2AX and other DDR proteins (90).

EVIDENCE FAVORING DIRECT RNA TOXICITY

A number of elegant animal- and cell-based studies have provided evidence of direct RNA toxicity (33,35,52,61,62). Most of those studies have identified specific candidate proteins, including purine-rich element binding protein A (Pur α), heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1), CUG triplet repeat RNA-binding protein 1 (CUGBP1), Sre-associated substrate during mitosis of 68 kDa (Sam68), etc., based on their interactions with the CGG-repeat RNA, and have demonstrated at least partial rescue of the wild-type phenotype by overexpressing the protein in the context of the expanded CGG-repeat RNA. In particular, in vivo studies demonstrated that Pur α and hnRNP A2/B1 were present in inclusions of FXTAS fly models, and that overexpression of Pur α or hnRNP A2/B1 clearly rescued the neurodegenerative phenotype (33,35), arguing for a sequestration-type mechanism.

Despite the evidence of sequestration, the high abundance of both Pur α and hnRNP A2/B1 raises another mechanistic possibility; namely, that whereas the initial effect of the rCGG repeat may not be directly related to the sequestration of the candidate protein, the subsequent overexpression rescues the wild-type phenotype through more general neuroprotection (33,35). This possibility has been reinforced for Pur α by the fact that the protein is not co-localized with intranuclear inclusions in either the CGG-knock-in or ectopic (cerebellar Purkinje cell) CGG-expressing mouse models, and is only intermittently detected in the inclusions in humans despite abundant cytoplasmic staining (33,51,62). The prospect of general neuroprotection was given more weight by the phenotypic rescue through the independent overexpression of heat-shock 70 kDa protein (Hsp70) (52). Indeed, there is abundant evidence that Pur α (63–65), hnRNP A2/B1 (66–69) and Hsp70 (70) each have general protective roles upon overexpression. It is important to note that these comments apply specifically to the issue of sequestration of these proteins—not their importance—in the pathogenesis of the human disorder, FXTAS.

Very recently, Sellier et al. (62) increased the number of candidate proteins for sequestration by transfecting expanded CGG-repeat plasmids into various cell models (e.g. neuronal, ovarian and kidney). One of their striking findings was that there is a sequential recruitment of proteins to intranuclear CGG mRNA: initially Sam68, MBNL1 and hnRNP G, and at later stages prior to cell death, the co-localization of a group of RNA-binding proteins (hnRNP A1, A2/B, C, D, E and H). The authors argued for the sequestration of Sam68 based on partial loss of function and consequent altered splicing patterns in FXTAS patients, a close parallel to the MBNL1 sequestration by the CUG repeat in DM1. Also noteworthy was their observation that the dynamics of aggregation of expanded CGG-mRNA with target proteins is consistent with the progressive enlargement of inclusions in mouse models of FXTAS (62). One remaining puzzle is why the inclusions in both human and mouse models of FXTAS are invariably solitary, whereas the aggregates observed by Sellier et al. (62) are multiple. Indeed, the mouse inclusions appear to originate as singlets, followed by enlargement, rather than through a process of coalescence. This distinction remains an interesting and open question.

The nature of the participation of CGG-repeat RNA in the pathogenesis of FXTAS has great bearing on the nature of the eventual therapeutic intervention(s). Notwithstanding the need for further clarification of the precise mechanism for RNA involvement, it is becoming increasingly clear that many of the features of cellular pathogenesis can be induced by the CGG-repeat region itself, to the exclusion of the FMRP protein coding region and/or the FMR1 promoter, or any obvious form of antisense transcript. This conclusion is based on the observation that both inclusion formation and other aspects of neuropathology can still occur in the absence of either native protein coding or promoter contexts (52,61). Sequestration certainly remains one possibility that ties directly to the CGG-repeat RNA. However, one cannot discount a direct role in which the expanded rCGG-repeat element acts as a trigger (Fig. 2), perhaps through its secondary structure, of various stress-response pathways (61,71,72). While a direct role of double-stranded-RNA-induced protein kinase (PKR) has been discounted (50), the principle of a trigger-type mechanism remains a viable alternative—or perhaps even co-pathogenic—to sequestration.

A BROADER VIEW OF RNA PATHOGENESIS IN FXTAS: TRANSCRIPTION IS REQUIRED FOR TOXICITY

While the preponderance of evidence points to an RNA-toxicity mechanism for FXTAS pathogenesis, it is more formally
correct to say that neurotoxicity requires transcription, and that the toxic insult is either post-transcriptional (i.e. directly RNA-mediated) or co-transcriptional (Fig. 2). Awareness of this latter possibility was raised by Entezam et al. (49), who demonstrated that CGG expansions in the premutation range recruit tel-angiectasia and Rad3 related protein (ATR), a DNA repair protein that responds to stalled DNA forks during replication, and which confers intergenerational repeat stability in a prema-

tation mouse model. It is therefore possible that DNA damage that may occur during the transcription of the expanded CGG-repeat region would recruit DNA repair proteins, and that the failure of the proper resolution of this process could, itself, have a negative impact in cell survival. In support of this concept, inclusions isolated post mortem from the brains of subjects with FXTAS do contain a phosphorylated form of H2A histone family member X (γH2AX) (32; unpublished data), a modified histone variant and a marker for DNA damage repair (DDR) (73). In this regard, the FXTAS inclusions also contain the nuclear intermediate filament protein isoforms, lamins A and C (32,72), which are known to become disorganized upon expression of the expanded CGG repeat (≏95 CGG; 61). More recent studies of dermal fibroblasts from FXTAS patients as well as unaffected adult (premu-
tation) carriers demonstrated similar abnormalities with the loss of the normal ring-like nuclear staining pattern (72). Such abnormalities may reflect a more general disruption of the integrity of the nuclear membrane architecture, which can in turn trigger a more generalized DNA damage response resulting in telomere shortening (74–76), cellular senescence and premu-
ture cell death (77).

AN EXPANDING FXTAS CLINICAL PHENOTYPE

Whereas FXTAS was initially thought of principally as a CNS disorder with a primary motor component, and involving loss of survival and dysfunction of both neuronal and glial cells (21,28), with concomitant white matter disease and global brain atrophy (19,26), it is now clear that the disorder is much broader, with significant non-motor components. A prominent feature of the broader neurodegenerative phenotype includes cognitive decline/dementia and related neuropsychiatric invol-

vement (4). In this regard, the intranuclear inclusions are most abundant in the amygdala and hippocampus (21,28), which are thought to impact emotional responsiveness, behavioral control and long-term memory. Amygdala dysfunction may underlie the social deficits in some premutation carriers (78–81). In fact, there is a negative correlation between hippo-
campus volume and anxiety in female carriers, with and without FXTAS (25). Loss of hippocampal volume may also be associated with mood disorders seen in FXTAS, particularly with depression and irritability (4,82). There is also clear evidence of hypothalamic-pituitary-adrenal axis dysfunction, with inclusions found in all three locations in humans and the KI mouse model (31,38,83). Finally, although fewer women than men will develop FXTAS, it does appear that women have a greater tendency to develop certain types of medical disorders than men, particularly autoimmune-type dysfunction, hypothy-
roidism and muscle pain (9); a compelling explanation for this paradoxical observation is still needed.

AN EMERGING VIEW OF FXTAS AS THE END-STAGE OF A PROCESS THAT BEGINS IN EARLY DEVELOPMENT

FXTAS is currently regarded as a late-onset neurodegen-
erative disorder; however, the underlying pathogenic process may begin at or before birth. The realization that an RNA-toxicity mechanism may have an early developmental component was based on an observation that in cultured hip-
campal neurons from post-natal day 1, premutation knock-in (KI) mice displayed abnormal dendritic arborization (decreased number of branches and decreased interbranch length) and decreased longevity in culture compared with neurons from wild-type littermates (71). Furthermore, abnor-
mal lamin A/C architecture, with loss of ring-like nuclear staining, is already present in embryonic fibroblasts from the KI mouse (72). These observations, coupled with elevated levels of FMR1 mRNA in young carriers of premutation alleles (24), lend support to the idea that the emotional and beha-

vioral difficulties, developmental delay, autism and autism spectrum disorder and increased seizure activity experienced by these children (78,84,85) are a consequence of an early developmental component of FMR1 mRNA-associated tox-

icity. The same possibility has been raised for FXPOI (11,13,40,42,57); as well as many of the neuropsychological and neuropsychiatric problems suffered by carriers in mid-


Thus, FXTAS may be a possible late outcome of an RNA-associated pathogenic process that actually begins very early in life, and which dictates not only the later degeneration of neural cells, but also their formation, development and long-term function.

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