



An explanation of the mechanisms underlying fragile X-associated premature ovarian insufficiency

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Received: 10 February 2020 / Accepted: 1 April 2020
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Abstract

Fragile X and fragile X-associated tremor-ataxia syndrome (FXTAS) are caused by mutations of the FMR1 gene. The mutations causing FXTAS can expand in a generation to a “full mutation” causing fragile X syndrome. The mutations causing FXTAS and the phenotype, fragile X-associated premature ovarian insufficiency (FXPOI), are referred to as the FMR1 premutation (PM). The objective of this paper was to formulate a theory to explain the Mechanism for FXPOI.

Recent research on fragile X syndrome and FXTAS has led to sophisticated theories about the mechanisms underlying these diseases. It has been proposed that similar mechanisms underlie FXPOI. Utilizing recent research on FXTAS, but a more detailed application of ovarian physiology, we present a more ovarian specific theory as to the primary mechanism explaining the development of FXPOI.

The FXPOI phenotype may best be viewed as derivative of the observation that fragile X PM carriers experience menopause an average of 5 years earlier than non-carriers. Women carrying the PM experience an earlier menopause because of an accelerated activation of their primordial follicle pool. This acceleration of primordial follicle activation occurs, in part, because of diminished AMH production. AMH production is diminished because of accelerated atresia of early antral follicles. This accelerated atresia likely occurs because the fragile X PM leads to a slowing of the rate of granulosa cell mitosis in some follicles.

Keywords FXPOI · Fragile X premature ovarian insufficiency · Fragile X premutation · Premature ovarian failure · Fragile X-associated premature ovarian failure · Fragile X-associated disease

Introduction

The most commonly known genetic cause of having menopause before age 40 or premature ovarian failure (POF) is due to a mutation in the promoter region of a gene coding for a protein that transports mRNA from the nucleus of a cell to its cytoplasm among other housekeeping functions [1]. The science underlying this mutation, an example of RNA-mediated nucleotide triplet repeat expansion diseases [2], has been

progressing rapidly. In part, this is because understanding these triplet expansion diseases has the potential to lead to treatments for a number of debilitating neurological and neuromuscular diseases [3–5].

Most people have a repetitive stretch of about 20 to 40 CGG nucleotides in the 5' untranslated region/promotor preceding the (historically named) fragile X mental retardation gene (FMR1) on the X chromosome [6]. This region of the X chromosome may expand or contract its triplet repeat number during meiosis because of errors in DNA replication, repair, and recombination. The fragile X syndrome occurs when an expanded number of CGG repeats exceed 200 (Fig. 1). When this occurs, the promoter region to the FMR1 gene becomes highly methylated, preventing polymerase binding to DNA at the initiation site and thereby silencing expression of the FMR1 gene [1]. Carrying this “full” mutation on an X chromosome causes moderate to severe mental retardation in approximately 100% of men and 50% of women [6].

In the past, the primary concern about a woman having an abnormal number (between 55 and 200) of CGG repeats was

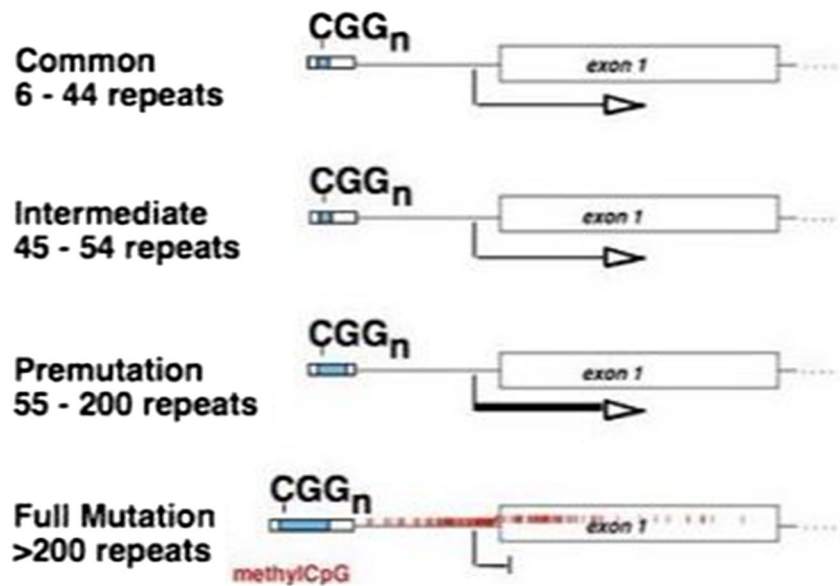
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Fig. 1 Four allelic classes of the human *FMR1* gene. For each allele, the size of the CGG repeat in the 5'-untranslated region of *FMR1* is indicated in blue in the figure. The level of transcription is indicated by the width of the arrow beneath each allele class. Note that there is increased transcription for premutation alleles and no transcription for full mutation alleles. (From Garber K, et al., *European Journal of Human Genetics* 2008; 16: 666–672)



that the mutation had the potential to expand its CGG repeat number during meiosis and that the woman could pass the “full mutation” to her children. For example, a woman carrying 70–79 CGG triplet repeats had a 31.1% chance of passing a full mutation to her progeny [7]. Subsequently, it became clear that individuals, who did not carry the full mutation, yet still had an abnormally high number of CGG repeats (historically labeled the “premutation”), frequently developed a late onset debilitating neuromuscular disease. This fragile X tremor ataxia syndrome (FXTAS) occurs in approximately 45.5% of carrier men and 16.5% of carrier women over age 50. Disease characteristics include intention tremor, gait ataxia, cognitive deficits, and brain atrophy [8, 9]. A common pathological finding for FXTAS is large ubiquitin-positive nuclear inclusions seen in the brain tissue. Evaluation of autopsied premutation (PM) carriers found these inclusions in all organs examined [10]. Studies in FXTAS mouse models suggest that it takes a period of time for these inclusions to develop [11]. A more detailed focus on the phenotype of people carrying a PM suggests that there is a range of impacts that may occur throughout their lives [9].

FXTAS mechanisms

The mechanisms underlying the phenotype FXTAS have been intensively studied using drosophila and mouse knock-in genetic models. The two best established theories for explaining the underlying mechanisms are based on the observations that PM carriers produce excess quantities (increased 2–8 fold in ovaries) of mRNA transcribed from the elongated triplet repeat and that PM carriers also produce toxic homopolypeptides secondary to aberrant transcription (using a non-traditional reading frame)(Fig. 2). The best studied of these proteins is fragile X polyglycine (FMRpolyG) [12, 13].

FMRpolyG is present in both the nucleus and cytoplasm of cells and is necessary for the formation of the nuclear inclusions seen in FXTAS [11, 12].

The excess aberrant mRNA, seen in FXTAS, fits the pattern of an “RNA gain-of-function disease.” The mechanism of such diseases involves the accumulation of mRNA, which then sequesters RNA and RNA-binding proteins, causing a functional depletion of these molecules within the cell. Toxicity is thought to occur over time secondary to interference with normal cell function if the cell is not able to compensate for the lost functional RNA and RNA-binding proteins [12, 14].

Recent elegant studies by Sellier et al. [11] suggest that the toxicity of FMRpolyG occurs because it binds to an isoform of lamina-associated polypeptide 2 (LAP2 β), as well as other lamina-associated proteins such as lamin A/C (essential components of the nuclear envelope). In sufficient quantity, this protein aggregation leads to a disruption of the nuclear architecture of a cell and to cell death [12, 15, 16].

POF (menopause before age 40) frequently occurs in premutation carriers. The reported incidence of POF varies with the study, but it is believed that approximately 10–28% of fragile X PM carriers develop POF [17–19]. This same population has a high incidence of menstrual cycle irregularities, hot flashes, depression, and infertility [20, 21]. This symptom constellation has been termed fragile X-associated premature ovarian insufficiency (FXPOI). The mechanisms underlying FXPOI are assumed to be similar to those identified for FXTAS [6, 20–22]. A recent investigation utilizing a knock-in mouse model speculated that FXPOI might be a reflection of dysfunction in the pituitary hypothalamic pituitary axis mediated through the impact of inclusion bodies containing mRNA and FMRpolyG found in the pituitary and other brain tissue of PM carriers [21, 23]. A more specific

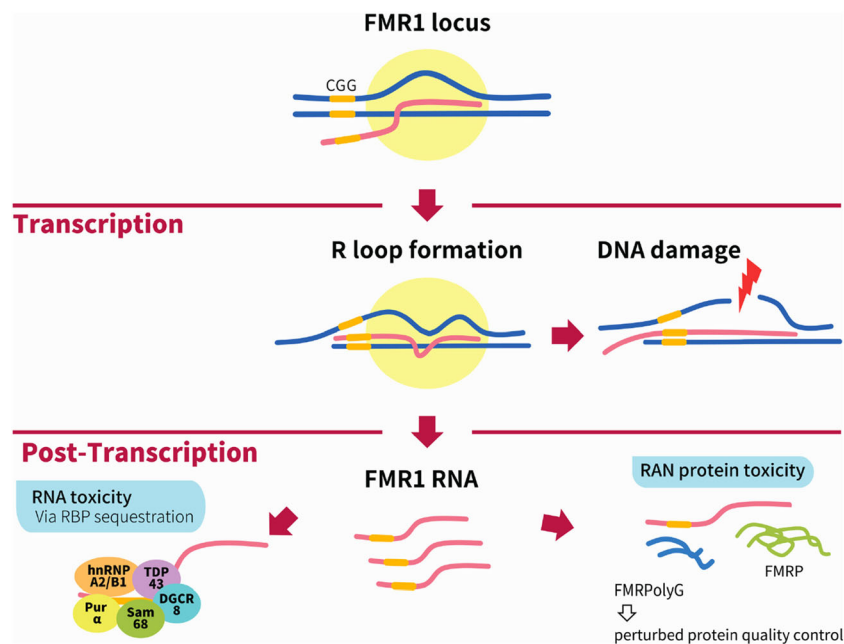


Fig. 2 Illustration of the main mechanisms of FXTAS pathogenesis. During transcription of the *FMR1* locus, the formation of RNA-DNA hybrid loops through GC interaction of the expanded CGG repeats (yellow bar) can activate a DNA damage response. The two main mechanisms linked to FXTAS pathology are post-transcriptional. In the RNA toxicity mechanism, RNA-binding proteins (RBPs) are sequestered by

the expanded CGG repeats (such as h2RNP A2/B1, Pur α , Sam68, TDP43, and DGCR8). In the RAN protein toxicity mechanism, the expanded CGG repeat induces AUG-independent translation of FMRpolyG. (FMRP is the normal protein product of *FMR1*.) (From Kong HE, et al. *Frontiers of Cellular Neuroscience* 2017; 11: article 128)

mechanism has been suggested by Lu et al. Using a transgenic mouse model, Lu suggested that FXPOI might occur secondary to the disruption of the Akt/mTOR signaling pathways in granulosa cells which play a role in cell cycle progression, DNA repair, and apoptosis [24].

In this paper, we present a more targeted explanation of the mechanisms underlying FXPOI. This theory is consistent with data obtained using genetically modified mouse models as well as data derived from the limited human tissue available. It is based on a more detailed application of what is known about ovarian physiology than has been used in the past (Table 1).

A quantitative approach to menopause and FXPOI

The average age of menopause (no menstrual bleeding for 1 year) varies slightly with the population examined (smoking, ethnicity, socioeconomic status, etc.) and the techniques used (age parameters, definition of menopause, compensation for mortality) with the average falling within the range of ages 49 to 52 [25]. For the purpose of this discussion, the findings of van Noord et al. are representative of other studies [26]. Van Noord et al. looked at a Dutch population

of 3756 women aged 72 to 86, who were enrolled in a population-based breast cancer screening program. The average age of menopause was 50.16 years with a standard deviation of 4.15 years. Assuming that the age of menopause in a population of women forms a normal distribution, the proportion of women having menopause prior to age 40 would be 0.71% (z score -2.45). The medical literature suggests that approximately 1% of women are thought to have POF [27].

Women, who carry a fragile X premutation, experience menopause an average of 5 years earlier than their age contemporaries [28, 29]. Assuming a similar distribution, a shift of the mean age of menopause by 5 years changes the z score for the proportion of women having menopause before age 40 to -1.24 . Statistically on this basis, one would expect approximately 10.8% of women carrying the PM to have POF.

The incidence of POF in PM carriers is estimated at 10–28% [17–19]. There is no large population-based estimate of the incidence of POF in PM carriers, and many studies utilize families identified by their having a family member with the (full) fragile X syndrome. Such study populations omit PM carriers who have no medical reasons to be identified and may not be representative of the larger population of PM carriers [7]. That is, they may overestimate the incidence of POF in PM carriers. The true proportion of PM carriers with POF is uncertain (certainly it may be higher than what was suggested

Table 1. Summary of proposed mechanism for the FXPOI phenotype in PM carriers

Event	Consequence	Reason	Support
Earlier menopause	-Increased premature ovarian failure -Premature ovarian insufficiency	Premature utilization of primordial follicles	-Menopause 5 years early -Normal size primordial follicle pool at birth
Lower AMH levels	Increased activation of primordial follicles	Loss of AMH suppression of primordial follicle activation	-Patients carrying the PM -Results from animal and cell culture models -Women carrying PM with PCO
Increased antral follicle atresia	Less AMH produced	Most AMH is produced by small antral follicles	Human and animal model ovarian histology
Impaired granulosa cell mitosis in some follicles	Increased follicular atresia during FSH driven growth stage	FSH binding (in more cells and with higher density of receptors) is essential to avoid triggering apoptosis	Established ovarian physiology
Specific impacts of a PM	FXPOI	-PM carriers fail to phosphorylate mTOR and thus impair mitosis in some cells -Increased mRNA may sequester RNA and RNA binding proteins faster than they can be replaced in rapidly dividing cells -FMRpolyG may impair mitosis by slowing down chromosome segregation or nuclear envelope development	Animal models with PM

here mathematically). However, the important observation is that much of the POF seen in PM carriers can be explained by the earlier menopause experienced in this group of women.

Perimenopause

The Massachusetts Women's Health Study was a longitudinal investigation of the perimenopause in approximately 2500 women over 5 years [30, 31]. Although many aspects of the perimenopause were examined, the start of the perimenopause was defined using the start of menstrual cycle irregularity (missed or shortened menstrual cycles). The study found that the perimenopause begins an average of 3.8 years prior to menopause. Continuing the above assumptions, the average age of the start of the perimenopause in PM carriers would be 8.8 years prior to the average age of menopause in non-carriers. Statistically, 37.1% of PM carriers should begin their perimenopause by age 40 ($z = -0.33$) and 6.3% by age 35 ($z = -1.53$). There are no identified clinically significant differences between the symptoms of FXPOI and the symptoms of perimenopause. The primary difference between FXPOI and the perimenopause is that the former occurs unexpectedly early in women who are PM carriers. Consequently, much of FXPOI can be attributed to fragile X PM carriers having menopause earlier than non-carriers.

Menopause occurs when the number of non-growing follicles (or primordial follicles) drops below a threshold, estimated to be approximately 500 or 1000 in each ovary [32–34]. Wallace and Kelsey and Depmann et al. constructed mathematical models of the decline of ovarian reserve with age based on data from the eight prior histological studies with direct follicle counts of 325 ovaries. Depmann et al. [33] excluded some of the cases included in the Wallace and Kelsey's model [32] and arrived at the lower oocyte threshold as defining menopause. Applying the Wallace and Kinsey model to the Massachusetts Women's Health Study data suggests that the average age of perimenopause begins when there are fewer than 5000 oocytes in each ovary. This approach to understanding menopause also implies that the wide range of ages at which menopause and perimenopause occur is a consequence of the wide range in the number of oocytes present at birth as suggested by these models.

Reason for loss of primordial follicles

An earlier average age of menopause in PM carriers suggests either a decrease in the number of primordial follicles present at birth or an increased utilization rate of primordial follicles during the pre-menopausal years. Follicle grading and follicle counts have been done in several PM rodent models. All models, in which this was evaluated, show that the primordial follicle population in the ovaries of PM carriers does not differ from wild-type mice [22–24, 35]. Thus the loss of oocytes from the ovaries of PM carriers must occur after primordial follicle activation. This is also true for mice that are homogeneous for the PM, for which later follicle losses were more severe [22, 36].

Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is primarily produced by granulosa cells in the primary to late antral follicle stages with highest expression in secondary follicles (two layers of granulosa cells) to early (≤ 4 mm diameter) antral follicles. Once follicles become FSH-dependent, AMH production by granulosa cells gradually decreases [36]. Most AMH is produced from a woman's early antral follicles, because collectively, they contain the majority of AMH-producing granulosa cells. For example, in the human by direct count, the number of granulosa cells in a 4-mm antral follicle is approximately two million cells [37].

Fragile X PM carriers have lower AMH levels than comparably aged women [38, 39]. Buijsen et al. showed a 9-fold increase in the number of atretic large antral follicles in the "Dutch" exCGG-KI (100–199 CGGs) mouse model [23]. Hoffman studied the (NIH) 130 CGG knock-in mouse at various ages and found an increased ratio of atretic to growing follicles with homologous mice having twice as many atretic follicles [22]. Dioguardi, also using the 130 CGG knock-in model, found a trend toward having a decreased number of large antral follicles and an increased number of atretic large antral follicles with differences becoming statistically significant in homozygotic mice. Both heterozygotes and homozygotes had a decreased number of corpus lutea [36]. Similarly, Lu et al., using a transgenic mouse model, showed that carrier mice had fewer growing follicles and that there was a decreased number of pups born per litter [24].

These models suggest that PM carriers have a decreased number of early antral follicles, which make the largest contributions to the AMH level. The increased antral follicle atresia in PM carriers causes a decrease in the granulosa cells available to make AMH. In addition, direct granulosa cell counts of follicles from the 130 GCC knock-in mouse model show a decrease in the number of granulosa cells compared to similar size follicles from wild-type mice [22]. Both of these factors may help explain the decreased AMH level found in human PM carriers.

Many factors play a role in the suppression and in the activation of primordial follicles [40]. One important suppressor of primordial follicle activation is AMH [41]. Culture of mouse ovaries with AMH showed a 40% decrease in the number of growing follicles compared to controls [42]. Activation of primordial follicles in human ovarian cortical tissue was suppressed using recombinant rat AMH [43]. Young AMH-null mice have an increased number of preantral and small antral follicles compared to wild-type mice. As they age, AMH-null mice have a significant decrease in the number of their primordial follicles compared to wild-type mice. Heterozygote AMH null mice have follicle numbers falling between wild-type and homozygous AMH null mice [44]. Pankhurst argues that AMH, as a suppressor of primordial

follicle activation, provides a long-term mechanism occurring in humans to extend the availability of an adequate number of healthy antral follicles to enable the selection of a good pre-ovulatory follicle. It does this by utilizing increased activation in the face of a natural decrease in the size of the primordial follicle pool [41].

The report by Tsafirir et al. of two patients who were PM carriers and also had polycystic ovarian syndrome (PCOS) was consistent with low AMH levels as a mechanism leading to diminished ovarian reserve in PM carriers [10]. Patients with PCOS have high AMH levels [45]. One would expect that PCOS patient's AMH levels would suppress primordial follicle activation and maintain their ovarian reserve. Both of Tsafirir's patients, with PCOS, carrying the PM, had a normal to high response to gonadotropins and produced 15–25 oocytes per cycle [10].

In summary, part of the mechanism for the development of FXPOI is the decrease in AMH levels seen in PM carriers which constitutes a decreased production of a major suppressor of primordial follicle activation. This contributes to accelerated primordial follicle activation compared to women with normal AMH levels.

Follicular atresia

Human PM carriers have more follicular atresia than non-carriers. This means, in the case of humans, that when a cohort of primordial follicles begins to grow, fewer of that cohort will become antral follicles from which a dominant follicle will be selected to eventually ovulate. Most follicle atresia occurs at the early antral stage of follicle development around the transition from being FSH-responsive to becoming FSH-dependent [46–51]. Significant granulosa cell death in primary, secondary, and preantral follicle stages is uncommon, and when it occurs, the pathway is normally autophagy rather than apoptosis [50]. Available PM carrier mouse models find that most follicle atresia occurs sometime after follicles reach the multi-laminar granulosa cell stage [22, 24, 35, 36].

Normal follicular development

The late preantral/early antral follicle is a complex entity. It contains an oocyte, a rapidly growing membrana granulosa, a basal lamina undergoing constant remodeling, and three to six layers of theca cells. Communication and increasing specialization is occurring in all components during follicle development. Theca layers and granulosa cell layers are differentiating so that the outer portions of these layers play a role in maintaining the structure of the follicle; whereas, inner portion of these layers become more endocrine. Most striking is the rapid multiplication of granulosa cells. In sheep, granulosa cells divide 12 to 14 times before developing an antrum [52]. In

humans, the number of granulosa cells increases from about four thousand to fifty million as the diameter of the follicle increases from 1 to 22 mm [53, 54]. In the human, the time period required for development from secondary follicle activation to becoming an FSH-dependent antral follicle (5 mm) is about 70 days [54]. The time period during which the follicle is FSH-dependent (5 to 20 mm) lasts about 14 days until ovulation [54]. Stated another way, in the early human follicle, the doubling time for granulosa cell mitosis is up to 250 h; whereas, once the follicle becomes FSH-dependent, the doubling time accelerates to around 50 h [54]. The increased loss of early antral follicles in PM carriers occurs around the time that granulosa cell mitosis begins to rapidly accelerate.

Although the average doubling time of granulosa cells in the membrana granulosa averages 50 h, for a subpopulation of cells, it may be much shorter. Studying bovine follicles, Lavranos et al. found that more than three quarters of granulosa cell mitosis during follicular growth occurs in the middle layers of the membrana granulosa [55].

Follicle atresia is a process, rather than a singular event. It requires granulosa cell apoptosis involving more and more of the membrana granulosa as the basal lamina folds in on itself; theca cells generally remain functional, the follicular antrum decreases in size, and eventually, the oocyte, itself, succumbs to apoptosis [46, 56, 57]. Successful IVF requires that follicles destined for atresia, and at times after the atresia process has started, can be rescued and further developed to provide a fertilizable oocyte. Atresia finally occurs, over time, when the balance of survival factors and death factors in the follicle/membrane granulosa moves sufficiently in favor of the later [47, 48, 51].

Essential role of rapid granulosa cell mitosis

In normal ovulatory women, follicle atresia begins after a dominant follicle has been selected (early antral follicle stage) and develops further in non-dominant follicles as that dominant follicle grows. To avoid atresia, a follicle has to expand its granulosa cell mass more rapidly than other follicles. This high rate of mitosis is driven by (exogenous or endogenous) FSH, which is also the strongest survival factor with respect to overcoming granulosa cell apoptosis [47, 48]. Estradiol is also essential for rapid mitosis but is produced secondary to aromatase production driven by FSH. Non-dominant follicles continue to survive as long as they can continue to adequately increase their granulosa cell mass. McNatty's research suggested that continued development of a follicle was associated with the number of granulosa it contained compared to what could be expected for a similar size dominant/healthy follicle [37]. As mitosis slows, the granulosa cell mass becomes less able to convert the available androgen substrate into estrogen. The fewer granulosa cells in a follicle, the higher the androgen to estrogen ratio [37]. Increasing androgens move the follicle

in the direction of increased granulosa cell apoptosis and eventually to follicle atresia [58].

It is likely that some granulosa cells in PM carriers have an impaired ability to undergo mitosis at an accelerated pace. As discussed above, a follicle, losing the race to increase its granulosa cell mass most quickly, defines a non-dominant follicle. The pace at which mitosis declines determines the timing of atresia within the timeline of normal follicular development. Using the 130 CGG mouse model, Hoffman et al. showed that similar size follicles in the PM model mouse had fewer granulosa cells than the wild-type mouse [22]. Quantitatively, this has been estimated to be 15% fewer [22]. That is, as the rate of granulosa cell mitosis becomes impaired, then some of a PM carrier's follicles will have difficulty increasing their granulosa cell mass rapidly. As this occurs, those follicles are forced down the path of accelerating atresia.

Specific impact of the PM

We propose that during the course of attempted accelerated mitosis, the dividing granulosa cells in some of a PM carrier's follicles are not able to accelerate their rate of mitosis enough to avoid the intrinsic pressure toward apoptosis. As the available FSH receptors fall behind (because of fewer granulosa cells available to express them) the availability of receptors in granulosa cells in other follicles, paracrine production of estradiol drops and theca androgen production continues unabated. The theca cells do not have a rapidly accelerating requirement for mitosis. Although androgen production is restricted by granulosa produced factors in the early follicle, by the late preantral follicle, it is driven primarily by the endocrine LH signal [57]. Eventually the degree of apoptosis occurring in granulosa cells in that follicle forces that follicle into atresia.

A study by Lu et al. suggests a cellular pathway to explain how this brake on accelerated mitosis of granulosa cells might occur [24]. Mammalian target of rapamycin (mTOR) is a serine/threonine kinase which is enhanced in granulosa cells during the M-phase of the cell cycle. The phosphorylated form of mTOR is enriched around the mitotic spindle and near the contractile ring during cytokinesis. Inhibition of the phosphorylation of mTOR reduced both granulosa cell proliferation and follicle growth during *in vitro* culture with arrest occurring in the G1 cell cycle stage [59, 60]. Lu et al. showed that mTOR and Akt phosphorylation is markedly reduced in granulosa cells in his PM mouse model [24]. In this mouse model, granulosa cell proliferation is reduced or slowed in PM carriers around the time of early antral follicle development. Slowing of granulosa cell mitosis would induce an environmental change in the follicles containing a threshold percentage of affected (mitosis impaired) granulosa cells. This change

could shift the balance between continued development and atresia toward an early atresia.

Increased mRNA

The hypothesis that the key factor causing FXPOI is a decreased ability to accelerate a follicle's rate of granulosa cell mitosis is consistent with accepted mechanisms for FXTAS. Mutant mRNA is produced in PM carriers and has been shown to cause sequestering of RNA and RNA-binding proteins [12]. These essential components of cell function must be compensated for by additional protein and RNA production if the cell is able to function normally. A cell might divide slightly more slowly as it works to compensate for these losses. The membrana granulosa may not be able to accelerate its rate of mitosis fivefold as it needs to after the transition to FSH dependence [54]. Elizur et al. showed that human PM carriers had increased levels of mRNA in their granulosa cells [61]. Allen et al. showed that that in women undergoing IVF for PGT, there was a correlation between the level of mRNA and the number of oocytes retrieved [62].

FMRpolyG

Rapid mitosis could also be disrupted through the primary mechanism advanced to explain FMRpolyG toxicity. FMRpolyG binds to LAP β which also binds to lamin A/C. These molecules stabilize the nuclear membrane and during mitosis facilitate correct chromosome segregation. Using neural cells transfected with expanded CGG-repeat plasmids, Arocena et al. were able to observe disruption of the lamin A/C architecture of the nuclear membrane [15]. Sellier et al, using induced pluripotent stem cells in culture which were differentiated into neurons, taken from a patient carrying a PM, showed that FMRpolyG was associated with the disorganization of lamin B1 in the nuclear lamina structure of those cells [11]. In the setting of granulosa cells, which divide more rapidly than neurons, FMRpolyG could impair mitosis.

Unlikely impact on theca cells

As noted earlier, a follicle is a complex entity with cellular components all playing an active concerted role in eventually producing a healthy fertilizable oocyte. Accepting that the PM slows granulosa cell mitosis and could contribute to accelerated atresia does not eliminate the possibility that the PM's effect on other components of the follicle could also be significant contributors to the FXPOI phenotype. The other primary cellular components are the theca cells and the oocyte.

Theca cells are unlikely to be a major component in the mechanism of accelerated antral follicle atresia. Both in human tissue and mouse models, inclusion bodies are found in ovarian stromal cells, but not in theca cells, in granulosa cells,

or in the oocyte [10, 22, 35, 36, 63]. These intra-nuclear inclusions are too big to leave the nucleus of a cell and, given their composition, cannot be metabolized by the stromal cell containing it [13]. It is well established that theca cells are recruited from the ovarian stromal cells [49, 57, 64]. Some form of selection must take place as the theca layer of a follicle is formed so that ovarian stromal cells with large intra-nuclear inclusion bodies are almost always excluded.

All PM carriers will have some ovarian stromal cells that do not express the PM. The theca cell layer and its underlying basal lamina have not completely developed until the late preantral stage of development prior to the follicle becoming FSH-dependent [49, 64, 65]. A follicle not able to form a full theca cell layer will be less capable of the endocrine expression and the granulosa cell mitosis required to become an antral follicle and therefore likely would be destined for atresia prior to antral formation [57]. Accelerated atresia of multi-laminar follicles in PM carriers has not been observed in animal models or human histology. Consequently in most humans, accelerated antral follicle atresia in the PM carrier is not likely due to inability to form a theca cell layer or by toxicity occurring within theca cells. However, some women with a high inactivation percentage of their normal X chromosomes or homozygote PM carriers could have more difficulty forming a healthy theca cell layer and producing follicles capable of full development because of difficulty in selecting/finding inclusion body-free stromal cells.

Unlikely impact on oocytes

On average, one half of the oocytes in PM carriers will carry the PM. Oocytes of human PM carriers increase their volume approximately 37 fold in the 3 months from primordial follicle activation to the preantral stage [66]. Much of the oocyte's volume increase is related to the increase in the mitochondria required to supply the ovulated oocyte with energy from ovulation to implantation. Dioguardi et al. in a PM mouse model has shown morphological mitochondrial abnormalities and an altered mitochondrial protein expression in oocytes [36]. The massive volume growth of the oocyte in early follicle development suggests that this is a time of very active transcription. Potentially the enhanced transcriptional demands could accelerate production of aberrant mRNA and proteins such as FMRpolyG. Neither of these potential problems for the oocyte provide a good explanation of why increased atresia occurs during antral stages since most of the change in oocyte volume occurs by the preantral stage (stage 3 follicle) [54, 67]. If the demands associated with increased mitochondrial production caused toxicity leading to atresia, that atresia would begin earlier in follicle development than was suggested by animal models.

Atresia could also occur if the toxicity secondary to the PM were sufficient to kill the oocyte. The limited data on PGT in

IVF patients, who are PM carriers, suggests that an oocyte carrying or not carrying the PM has a similar chance of becoming a blastocyst. For example, Reches et al. successfully analyzed 250 embryos (from 14 patients undergoing 47 cycles) and found that 49.6% carried the abnormal allele and 40.5% carried the normal allele [68]. Platteau et al. performed day 3 biopsies on 61 embryos (from 9 patients undergoing 17 cycles) and found that 44.3% were unaffected and 40.9% carried the pre- or full mutation [69]. If oocytes carrying the PM were dying in large numbers at the early antral stage, there would be a significant difference in the number of blastocysts which do or do not carry the PM. An oocyte carrying the PM appears to be as likely to emerge from the IVF process and become a blastocyst as a normal oocyte. Thus the PM is not likely to be directly killing oocytes.

However, perhaps there is a lower level of toxicity directed at the oocyte which does not impair the oocyte enough that it cannot still be rescued by exogenous FSH as used in IVF. However, if the oocyte has been damaged, that damage must show up by impairing some key post-ovulatory function. As noted above, it does not seem to impair the ability of the fertilized oocyte to become a blastocyst. Carrying the PM also does not impair the ability of the oocyte to mature or to become fertilized [19, 70]. This finding is especially striking as the oocyte/embryo is believed to be transcriptionally silent prior to fertilization until after several cell divisions [65], and if essential RNA or RNA-binding proteins were being functionally deactivated, the oocyte would likely have limited ability to compensate for them.

Variable expression of the FXPOI phenotype

If being a PM carrier leads to the disruption of mitosis of granulosa cells, it raises the concern that some PM carriers might only ovulate rarely. Fortunately, the impact of the PM on granulosa cell mutation is not a universal ovarian event. The proportion of granulosa cells that express the PM differs greatly from follicle to follicle. The primary reason for this is that the granulosa cells in a single follicle are oligoclonal. A small number (3 to 5 cells) of pre-granulosa cells will surround the oocyte to form a primordial follicle during fetal life. All of the granulosa cells in a follicle will be descendant from these few cells [71, 72]. Elementary statistics suggests that some follicles will have no granulosa cells carrying the PM. Some follicles will have all granulosa cells expressing the PM. Whether or not a specific follicle has a large or small proportion of its granulosa cells expressing the PM will vary significantly. This variability may also be magnified in individual women by variations in the inactivation of the X chromosome carrying the PM. Since imprinting is maintained through mitosis, if a pre-granulosa cell that forms a primordial follicle has an inactivated X chromosome that carries the PM, then none of the millions of cells in that follicle descended from that cell

will express the PM. The literature suggests that on average the proportion of X chromosomes carrying the permutation that are inactivated is around 50% [6], but there will always be women who will have a much larger or smaller proportion of their X chromosomes carrying the mutant gene inactivated. For example, Sullivan et al. evaluated the PM inactivation of five PM carriers with POF and found a PM inactivation ratio of 30–63% [73].

Summary

PM carriers develop FXPOI because of impaired granulosa cell mitosis in some of their follicles. Failure to adequately phosphorylate mTOR is likely a key part of this impairment, and any impact of the PM on slowing mitosis of granulosa cells is a potential contributor. Impaired mitosis of granulosa cells moves the follicle in the direction of atresia. This atresia becomes more likely when the follicle development requirement of accelerated mitosis occurs, which is around the time of antral formation and FSH dependence. Increased atresia of antral follicles leads to decreased production of AMH. Decreased AMH decreases the inhibition of primordial follicle activation by AMH. Increased primordial follicle activation eventually leads to decreased ovarian reserve and an earlier menopause. An earlier menopause in PM carriers is mathematically associated with a significant incidence of POF and a common early presentation of pre-menopausal symptoms.

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