Characterization of the full fragile X syndrome mutation in fetal gametes

Henry E. Malter¹, Jane C. Iber¹, R. Willemsen², Esther de Graaff², Jack C. Tarleton³,⁵, J. Leisti⁴, Stephen T. Warren¹ & Ben A. Oostra²

Fragile X syndrome results from the expansion of the CGG repeat in the FMR1 gene. Expansion has been suggested to be a postzygotic event with the germline protected. From an analysis of intact ovaries of full mutation fetuses, we now show that only full expansion alleles can be detected in oocytes (but in the unmethylated state). Similarly, the testes of a 13-week full mutation fetus show no evidence of premutations while a 17-week full mutation fetus exhibits some germ cells with attributes of premutations. These data discount the hypothesis that the germline is protected from full expansion and suggest full mutation contraction in the immature testis. Thus, full expansion may already exist in the maternal oocyte, or postzygotic expansion, if it occurs, arises quite early in development prior to germline segregation.

Fragile X syndrome is a common form of mental retardation caused by the expansion of a CGG repeat within the 5'-untranslated portion of the FMR1 gene¹,². This repeat is normally polymorphic in length and content, exhibiting a mode of 30 triplets (normal range of ~6-60 repeats) frequently interrupted by 1 to 3 AGG triplets³-⁵. In contrast to these normal alleles, individuals with fragile X syndrome exhibit greater than ~230 CGG repeats with an average of nearly 800 triplets; these are referred to as full mutations⁶ and are abnormally methylated and transcriptionally silent⁷,⁸. Nonpenetrant carriers of fragile X syndrome display an intermediate number (~60-200) of FMR1 repeats⁹. Whereas normal FMR1 alleles are stably transmitted to offspring, the carrier alleles, called premutations, are distinctly unstable upon transmission, with the parental premutation changing to typically longer arrays in children. This instability shows a distinct parent-of-origin effect: expansion to full mutations occurs in maternal rather than paternal transmission¹⁰,¹¹. The mechanism(s) responsible for this parent-of-origin effect has yet to be elucidated.

Several hypotheses have been put forth to account for the exclusive maternal transmission of fragile X syndrome: i) a maternal germline event leads to transmission of the full mutation allele or ii) mitotic events result in expansion of the inherited maternal premutation to the full mutation in the early embryo. Although the strong parent-of-origin effect favors a meiotic basis for this expansion, there is considerable acceptance of a postzygotic expansion of the maternally inherited premutation based on the observation by Reyniers et al.⁹ that adult fragile X males who exhibit the fully expanded FMR1 repeat in all tested somatic cells, show only premutation length repeats in their sperm. Although testicular selection against full mutation sperms was considered, the normal fertility of male Fmr1 knockout mice appeared to disprove this theory¹⁰. Moreover, the typical mosaicism in individuals carrying the full mutation, manifested by multiple alleles with distinct FMR1 repeat lengths, was consistent with mitotic expansion¹¹. Together with data showing that expanded repeats, regardless of the degree of mosaicism, were stable during cell culture and apparently in vivo during adult life, the concept arose that repeat expansion must occur during a fixed window between 5 and 20 days postnatal development¹²,¹³. A key facet of this model is that this developmental window follows the segregation of the primordial germline from the embryo proper, thus sequestering the germline from the somatic tissue¹⁴.

Despite the attraction of the mitotic model for explaining a number of puzzling aspects of the genetics of fragile X syndrome, it still requires one to invoke an undefined parental imprint on the maternal premutation which expands in the embryonic somatic tissue whereas a paternal premutation does not.

The mitotic model of repeat expansion clearly predicts that the embryo carrying a full mutation derived from a maternal premutation should only exhibit premutation alleles in gametes since the sex of the embryo is indeterminate at the point of primordial germ cell (PGC) segregation. It is shown below through critical analysis of intact gonadal tissue, that germline cells of full mutation fetuses of either sex do not show evidence of premutations. These data indicate that the mitotic model of FMR1 repeat expansion with a protected germline is untenable and that the presence of premutation sperm in full mutation males likely reflects a selective advantage for spermatogonia which had undergone mitotic contraction of repeat length.

Fetal ovary studies
We obtained tissues from a female fetus of 16 weeks gestation carrying the full FMR1 mutation on one of the two X chromosomes. The X chromosome carrying the full mutation was inherited from a premutation mother who carried a FMR1 allele with 70 repeats without evidence of mosaicism. DNA was prepared from various tissues and subjected to Southern blot analysis to assess the FMR1 repeat length and methylation status⁷. In the normal allele, EcoRI liberates a 5.2-kb fragment containing two adjacent BssHII sites, which, when the DNA is unmethylated, results in a 2.4- and 2.8-kb fragment. The latter genomic fragment contains the CGG repeat and is shifted to higher molecular weights when the repeat is expanded. In normal females, two patterns are seen reflecting the active X chromosome (unmethylated repeats) and the inactive X (methylated repeats) that is sequestered during fetal development.

¹Howard Hughes Medical Institute and Department of Biochemistry and Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA. ²Department of Clinical Genetics, Erasmus University, Rotterdam, The Netherlands. ³Greenwood Genetics Center, Greenwood, SC, USA. ⁴Oulu University, Finland. ⁵Current address: Mission Genetics Center, Asheville, NC 28803, USA. Correspondence should be addressed to S.T.W. e-mail: swarren@bimcore.emory.edu
would be consistent with observations that many X-linked loci, including the normal \( FMR1 \) gene, typically show unmethylated alleles of both X chromosomes in oocytes. To confirm the presence of oocyte DNA in the ovarian sample, the ovarian DNA and control fetal DNA were cleaved with \( EcoRI/HpaII \) and analysed by Southern analysis with a probe derived upstream of the X-linked \( P3 \) gene. Hybridizing this \( P3 \) probe to \( EcoRI/HpaII \)-digested DNA will reveal a diagnostic 2-kb band specific to ovarian DNA in addition to other bands common to DNA of all female tissues. This 2-kb band reflects cleavage of a unique \( HpaII \) site unmethylated in fetal oocytes between 13 and 21 weeks gestation. Analysis of the fetal DNA showed the clear presence of an oocyte-specific 2-kb band in the ovarian sample that is absent in all other tested tissues, indicating the presence of oocyte-derived DNA. Furthermore, the intensity of this 2-kb fragment is \( \sim 19\% \) of the total signal by densitometry, which correlates well with the estimates of oocyte mass relative to ovarian mass. The intensity of the 4.7-kb band is \( \sim 10\% \) of the total peak signal by densitometry. This agrees with the postulated \( \sim 20\% \) oocyte-specific signal since the 4.7-kb band is derived from the single expanded X chromosome only. Therefore, it is likely that the 4.7-kb fragment is derived from oocyte DNA that contains an \( FMR1 \) allele with the full mutation expanded to a very similar degree compared to that seen in various other tissues. However, unlike those tissues, this allele is unmethylated at one or both of the \( BssHII \) sites.

Although Southern analysis revealed the full fragile X mutation and the normal paternal \( FMR1 \) allele (that is, the 2.4-, 2.8-, and 5.2-kb bands), there was no obvious sign of a premutation. As the full mutation was derived from a maternal premutation of 70 repeats, current theories would predict premutation alleles in the germline. As \( \sim 19\% \) of the ovarian sample is derived from the germline and about half of the oocyte DNA can be accounted for as an expanded but hypomethylated allele, we carried out a sensitive PCR study to search for evidence of premutation alleles (see Methods). A 1:12 mixture of premutation to full mutation DNA (\( \sim 8\% \) premutation) resulted in a clear premutation band (Fig. 1c). Upon overexposure, as little as a 1:24 mixture (\( \sim 4\% \) premutation) revealed a faint premutation band (data not shown). PCR analysis of the fetal ovarian DNA showed the paternal allele of 29 repeats and full mutation bands in excess of 450 repeats with no evidence of premutation-sized alleles (Fig. 3, ovary I) even after prolonged exposures. In addition, another fetal ovary sample from a full mutation heterozygote of 17 weeks gestation became available late in this study. Although there was insufficient DNA to fully evaluate this ovary, PCR studies similarly showed only the paternal allele (29 repeats) and the full mutation with no evidence of any premutation fragments (Fig. 1c, ovary II). This is despite the fact that the mother carried a premutation allele. Moreover, this ovarian tissue showed similar evidence for hypomethylated full expansions in oocytes based upon \( EcoRI/EagI \) digestion (data not shown). Although we cannot exclude a premu-

**Fig. 1** a, Southern analysis of tissues from a 16-week fetus reveals the presence of only normal and full mutation \( FMR1 \) alleles in the female germline. The ovary sample, containing both oocytes and somatic tissue, reveals only normal and fully expanded repeat alleles in the methylated and unmethylated states with no evidence of premutations. b, Southern analysis of the 16-week full mutation female fetus confirms oocyte-specific DNA in the ovarian sample. A 2-kb fragment is unique to unmethylated oocyte DNA. c, Polymerase chain reaction analysis of fetal germline reveals only normal and fully expanded \( FMR1 \) alleles. In the 16-week fetal ovary sample (ovary I, lane 5) and a 17-week fetal ovary (ovary II, lane 6), products were obtained only from the normal and full mutation alleles. In a 13-week (lanes 7,8) and a 17-week (lanes 9,10) male fetus, only full mutation products were obtained in testes and skin samples although a faint band at 135 repeats could be appreciated in the 17-week fetus upon longer exposure. Control templates consisting of male premutational and full mutation DNA, alone (lane 1, 2) or mixed at 1:24 and 1:12 ratios (lanes 3, 4) (see Methods).
**Fig. 2** FMRP is not expressed in the germ cells of a full mutation 13-week testis and expressed only in a limited subset of germ cells in a full mutation 17-week testis. Thick sections taken from a, a normal 13-week testis and from b, c, the 13- and 17-week full mutation testes were immunostained with a FMRP-specific antibody. At 13 weeks FMRP is abundant in male germ cells (a). In contrast, the full mutation 13-week testis shows no FMRP expression (b, PGCs indicated by arrowheads) while the 17-week testis is mostly negative for FMRP, including most PGCs (c, arrowheads), however a limited subset of PGCs now express FMRP (c, arrows).

**Fetal testes studies**

To extend this study to the early male germline, we evaluated two male fetuses at 13- and 17-weeks gestation. Analysis of somatic tissue of both fetuses showed the full mutation in the fully methylated state without obvious mosaicism of fragments less than 200 repeats (data not shown). The mothers of both fetuses carried premutation alleles of 80 and 95 repeats, respectively. Accurate assessments of human male germ cell abundance have not been reported during the developmental time points associated with these male fetuses. In the rat, PGCs make up 19–25% of the testicular tissue during the same relative developmental period, although a much smaller percentage of PGCs is frequently cited in humans.

Southern analysis showed only methylated full mutations in testes and brain without evidence of premutations (data not shown). However, given that a minority of testicular tissue is PGCs at this point in development, we utilized the PCR approach above to detect premutation alleles in PGC-derived DNA. Both fetal testis samples had full mutation patterns similar to their somatic tissue (Fig. 1c). On prolonged exposure no predominant alleles <200 repeats were observed in testis or skin DNA although the 17-week fetus did show a faint band estimated to be ~135 repeats.

These data together with the fetal oocyte data support the notion that the majority of PGCs derived from full mutation fetuses do not carry premutation alleles. To more carefully evaluate this, we performed immunohistochemistry upon testicular tissue sections from the above fetuses with antibody against the FMRP1 protein, FMRP. The rationale is that i) FMRP is normally expressed in PGCs in fetal testes, ii) premutation cells, including those of adult testes, also express FMRP, and iii) male somatic cells that carry the fully expanded FMRP gene do not express FMRP. Thus the presence of premutation alleles in the fetal PGCs of these fetuses should result in antibody reactivity.

FMRP is abundantly expressed in PGCs in normal 13-week fetal testes (Fig. 2a). In contrast, the section from the 13-week full mutation fetus shows complete absence of FMRP staining in a number of otherwise normal appearing PGCs (Fig. 2b, arrowheads). This strongly supports the conclusion based on the PCR data that fetal testes, including the PGCs, do not contain premutation alleles at 13 weeks gestation. However, the 17-week testis shows some evidence of FMRP expression. Although the majority of the testis is FMRP-negative, these data clearly show some PGCs expressing FMRP (Fig. 2c, arrows) while other PGCs (arrowheads) remain negative. This is consistent with the faint premutation band observed in the 17-week fetal testis. Although a constitutional, low level of mosaicism for premutation alleles in the 17-week fetus cannot be ruled out, no FMRP expression was observed in somatic neuronal tissue similarly examined (data not shown). Taken together with the absence of expression in the 13-week testis, these data suggest that a reduction of full mutation alleles is responsible for premutation sperm of adult patients, rather than the maternal premutation being maintained in the male germline.

**Discussion**

This study contradicts previous models in which the germline was sequestered from the expansion process and indicate instead that premutation expansion must occur either during germline development in the transmitting mother or in the zygote/embryo prior to separa-
Fig. 3 A model for the repeat expansion process at FMR1. a, The mitotic model where the maternal premutation is present in the oocyte and later expands in the early embryo. b, The meiotic model where the oocyte of a premutation mother carries the expanded full mutation and subsequent contraction occurs in the early embryo. Around day 5 the primordial germ cells separate from the embryo proper, containing the full mutation allele under either model. Later, in the immature testes, some PGCs undergo contraction to premutations. These PGCs later predominate in the mature testes through a selective process. Such instability and/or selection does not occur in the oocyte.

Reproduction of the germ line (Fig. 3). If the maternal premutation remains unexpanded in the oocyte, there must be a strong bias towards full expansion limited to the very early rounds of DNA replication and division in the embryo, since germ line separation has often been suggested to occur as early as day 5 in the human, although the exact timing of this event has not been rigorously established. Mosaicism, under this scenario, would develop by individual variation in the degree of each expansion event and then become fixed when the expansion window closes (Fig. 3a). Depending on the strength of the expansion bias and length of the expansion period, somatic cell lineages could retain the original maternal allele resulting in the premutation-range alleles sometimes observed in full mutation FMR1 mosaics. This gametic premutation model still requires an unknown imprinting phenomenon which either predisposes a maternally derived allele to expansion or protects the paternally derived allele from this process.

In the second scenario, maternal transmission of fully expanded repeats would derive from expansion during female germ line development/genesis (Fig. 3b). In this case, the maternal transmission bias could result from sex-specific differences in gametogenesis leading to the production of full expansion oocytes in carrier females and premutation sperm in carrier males. This is compatible with the observation that mature spermatozoa with FMR1 full mutations have never been observed in males harbouring full mutations in their somatic tissue. If the zygote begins development with a fully expanded FMR1 repeat, then somatic mosaicism must result from reductional repeat instability limited to a window in early development as discussed above. Such reductional instability may be even more pronounced in the fetal testes. Sensitive PCR analysis showed that premutation alleles are absent or rare in such testes. This conclusion is strongly supported by the complete absence of FMRP expression in the testes of the 13-week full mutation fetus in contrast to the abundant expression seen at 13 weeks in a normal testes. However, the observation remains that mature spermatozoa from fragile X full mutation adult males harbour only premutations. The most likely hypothesis is that repeat contraction occurs in male germ cells sometime after week 13 of development and this combined with selection for the FMRP-expressing germ cells results in the production of mature premutation spermatozoa. This contraction hypothesis is supported by evidence from FMRP expression that in the 17-week fetus some but not all germ cells harbour premutations. This time point falls within a period of mitotic germ cell proliferation that continues until about week 20 (ref. 29). Although we cannot completely rule out constitutional mosaicism for premutation alleles in this fetus, such alleles were not present in neuronal tissue. It is therefore possible that there is a unique contraction mechanism associated with male germ cell development. It is known that contraction of FMR1 premutation alleles is more common upon male than female transmission. Moreover, other trinucleotide repeat loci frequently exhibit marked instability associated with paternal versus maternal transmission. Also, a strong paternal bias exists for the inheritance of de novo chromosome structural rearrangements, suggesting male gametogenesis is prone to instability. The subpopulation of FMRP-expressing premutation germ cells might gain a selective advantage during the explosive growth of the germine which achieves >85% of testicular mass in the adult. Indeed, markedly enhanced levels of FMRP have been noted in type A1 spermatogonia in the immature testes. From the limited evidence available, the premutation alleles in spermatozoa are unique and do not reflect the usual mosaicism observed in somatic tissue.
Our studies also address a long-standing debate about the methylation status of \( \text{FMR1} \) during the expansion process, providing strong evidence that the fully-expanded \( \text{FMR1} \) locus in fetal oocytes is unmethylated. This is in contradiction with a previous hypothesis that the proximal cause of fragile X syndrome was a failure to erase the maternal imprint, presumably marked by methylation, from this locus.\(^{36,39}\)

Although we cannot rule out methylation at locations other than the \( \text{BstIII} \) sites analysed, oocyte hypomethylation at this locus is consistent with the behaviour of other \( X \)-linked genes including the normal \( \text{FMR1} \) gene, which are normally methylated on the inactive \( X \) but are consistently hypomethylated on both \( X \) chromosomes in oocytes.\(^{19,20}\) Although our survey is limited, taken together with the observation that extra embryonic tissue of full mutation fetuses is commonly hypomethylated, it strongly suggests that full repeat expansion precedes abnormal methylation at \( \text{FMR1} \) (ref. 7). This is consistent with the fact that both extra embryonic tissue and PGs migrate from the generally unmethylated epiblast prior to the wave of de novo methylation that occurs in the embryo proper.\(^{19,20}\)

In summary, massive expansion of the CGG repeat associated with fragile X syndrome does not occur following germ cell segregation from the embryo proper, but instead is likely the result of contraction of the full expansion in the fetal testes with subsequent selection for premutation-bearing spermatogonia. Indeed, expansion may have already occurred in the maternal germline or, if embryonic, it occurs very early in embryogenesis, prior to de novo methylation. Further delineation between the genetic or embryonic expansion models will require a determination of repeat status in the oocytes of premutation carriers. However, given the difficulty in acquiring such specimens, these experiments may not be immediately forthcoming.

**Methods**

Southern blot and PCR analysis. Total genomic DNA was prepared from all tissue samples via proteinase K digestion and phenol extraction. For Southern blot analysis, 8 \( \mu \)g of each DNA sample was digested with 
\( \text{EcoRI} \) and \( \text{BstIII} \) or \( \text{HpaII} \) followed by electrophoresis on a 1.2\% agarose gel. The resulting gel was transferred to a membrane followed by hybridization with either the \( \text{FMR1} \) probe or 3 specific probes.\(^{40,41}\) The 2.6 kb \( \text{FMR1} \) gene probe was a PCR product obtained by amplification of human genomic DNA with primers specific to the \( \text{FMR1} \) gene: P3E: \( 5'-\text{GCAGTCATTAGCGCCGGTTCG}-3' \), P3F: \( 5'-\text{GCGGCTGTAGATGGCGAGACG}-3' \), P3F: \( 5'-\text{GCGGCTGTAGATGGCGAGACG}-3' \). Blots were washed and exposed to X-ray film to produce autoradiographs. Densitometry was performed from original autoradiographs on linear range signals using video imaging and the LYNX 5000 system (Applied Densitometry).

PCR analysis was performed essentially as described.\(^{42}\) The \( \text{FMR1} \) CGG repeat was amplified using primers, C and F, flanking the repeat. Control PCR templates were derived from a premutation male (95 repeats), a full mutation male (450+ repeats), and a male (1.12 and 1.24 mixtures of the pre/full mutation male templates. Total DNA concentration was approximately 75 ng total for each reaction. The products were resolved through polyacrylamide gels that were subsequently blotted and probed with radiolabelled CGG oligonucleotide.\(^{42}\)

**Immunohistochemistry**

The fetal testes were embedded in Tissue-Tek (Miles Laboratory) and frozen in liquid \( N_2 \). Thick (7 \( \mu \)m) sections were immunostained for \( \text{FMRP} \) as described previously\(^{43}\) using the indirect immunoperoxidase technique.

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