

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Results

5.1.1. Primordial follicle counting

Hematoxylline-eosine staining of paraffin sections of ovaries from P6, P25, ~20wk and ~40wk mice was performed to study the growth and development of follicles in premutation mice.

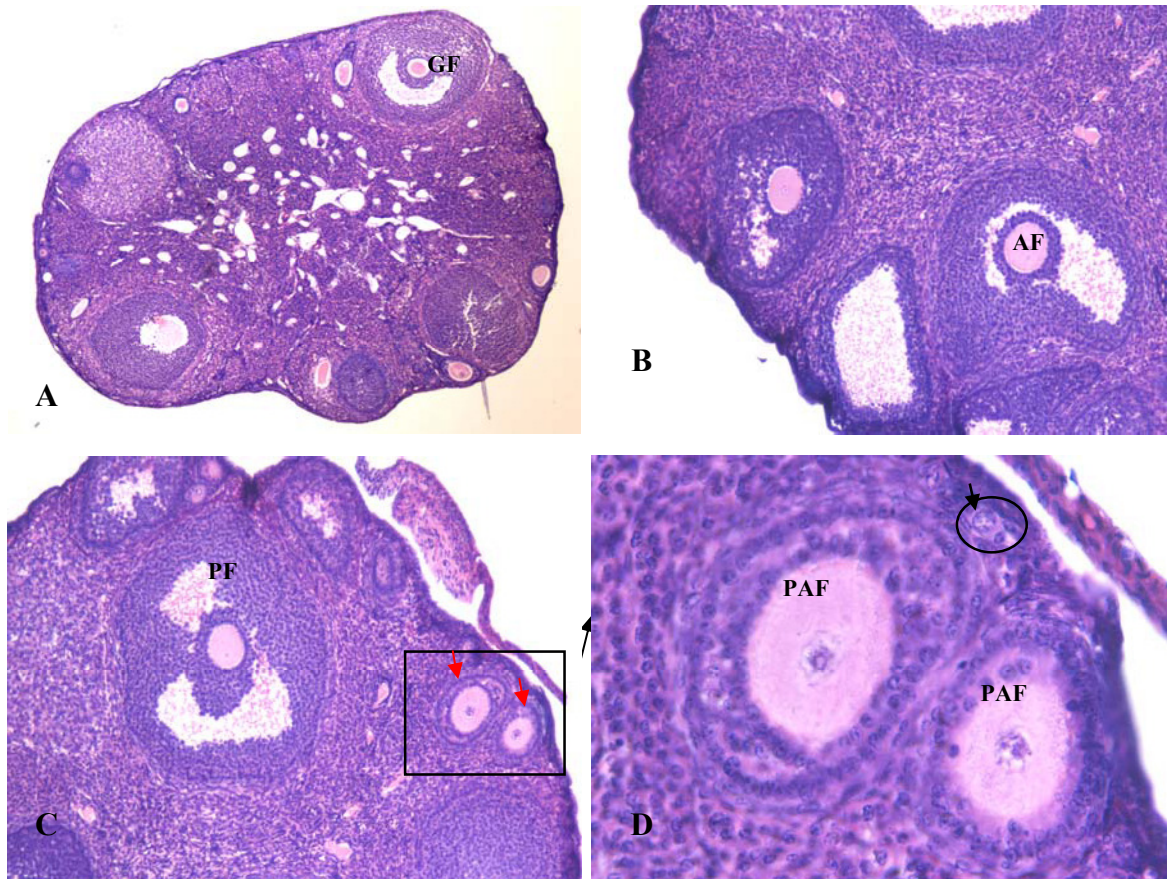


Figure 5.1. Photomicrograph of ovarian histology of ~20wk premutation mice using Hematoxyllin-Eosine (HE) staining. Magnification 40x for A, 100x for B, C and 400x for D. The pictures are showing all of stage of ovarian follicles. (A) showing graafian follicle (GF) determined by size with transparent vesicle (antrum), cumulus ophorus and its position which protrudes from the surface of the ovary. (B) showing antral follicle (AF) determined by

diameter $<310\ \mu\text{m}$ with antrum and its position near cortex of ovary. (C) Showing preovulatory follicle (PF) determined by diameter $>370\ \mu\text{m}$ with antrum and its position near cortex of ovary and preantral follicles (red arrow). (D) Showing Primordial follicle (black arrow and circle) recognized by the mean diameter of the follicle ($<20\ \mu\text{m}$), partially or completely encapsulated by one layer of flattened squamous pregranulosa cells and consist of oocyte, and preantral follicle (PAF) recognized by its diameter $100\text{-}220\ \mu\text{m}$ without transparent vesicle (antrum).

Ovarian histology of premutation mice were compared to wt mice. All stages of follicle were showed in ovaries of adult ($\sim 20\text{wk}$) premutation mice, showing all stages of follicles (See Figure 5.1), and from P6 ovaries only primordial follicles is showed (See Figure 5.2).

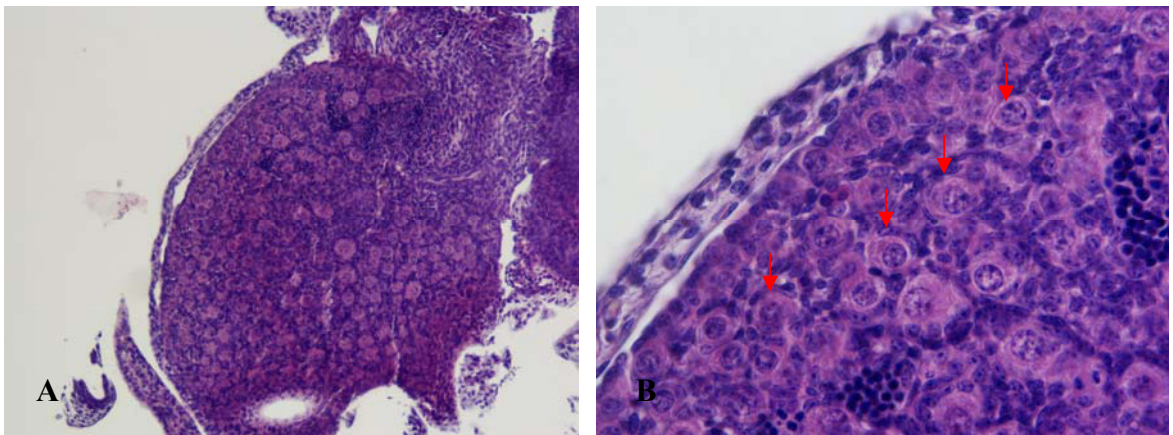


Figure 5.2. Photomicrograph of ovarian histology of P6 premutation mice. (A) showing only primordial follicles in ovary of P6 mice. (B) showing primordial follicle (red arrow) recognized by the mean diameter of the follicle ($<20\ \mu\text{m}$) and morphological criteria (primordial follicles are in rest and consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells). Magnification $\times 100$ (A) and $\times 200$ (B). Hematoxylline-Eosin Staining.

The number of primordial follicles were quantified in ovaries from wt and premutation mice at different ages to study the effect of expanded CGG repeat on the folliculogenesis. The means of number of primordial follicles from P6, P25, $\sim 20\text{wk}$, $\sim 40\text{wk}$ mice are depicted in table 5.1. There is no significant difference was detected in the number of primordial follicles between wt and premutation mice in P6 and $\sim 20\text{wk}$ ($p=0.052$ for P6 mice, $p=0.515$ for $\sim 20\text{wk}$ mice). However

at P25 and ~40wk premutation mice the number of primordial follicles was significantly higher compared to wt mice ($p=0.018$ for P25 mice, $p=0.006$ for ~40wk mice).

Table 5.1 Primordial follicle number premutation and wt mice in several ages

Age	Primordial Follicle Number (Mean \pm SD)		p value
	premutation mice	wt	
	P6	4019,09 \pm 1474,29	
P25	1232,83 \pm 195,91	757,71 \pm 377,90	0,018 ^{*)}
~20wk	718,20 \pm 377,47	574 \pm 99,88	0,515 ^{**)}
~40wk	318,86 \pm 112,27	161,78 \pm 84,80	0,006 ^{*)}

^{*)} T-Test

^{**)} Mann Whitney U Test

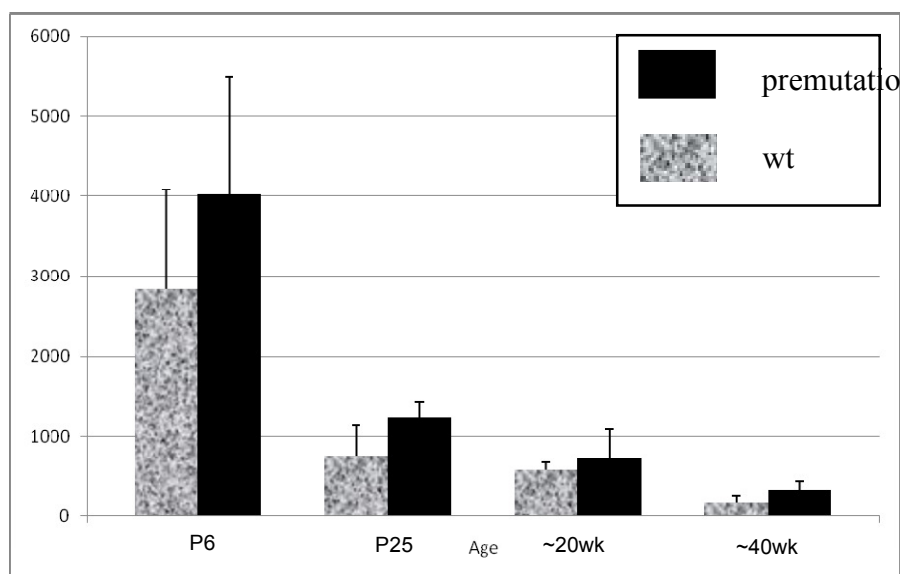


Figure 5.3. Primordial follicle population in P6, P25, ~20wk and ~40wk premutation, and wt mice. The difference of primordial follicle number was statistically significant in P25 and 40wk premutation mice compared to wt. but it was not in P6 and 20wk premutation mice.

Following this results, Primordial follicle depletion in several age were counted to determine the progressivity of primordial follicle depletion in premutation mice and wt. The percentage of primordial follicle depletion are depicted in Table 5.2 and Figure 5.4. Primordial follicle depletion in premutation mice from P6 to varying age are lower than wt mice.

Table 5.2. Percentage of primordial follicle depletion from P6 to varying age in premutation and wt mice.

Age	Reduction of Primordial Follicle numbers (%)	
	premutation mice	wt
P6-P25	69,32	73,27
P6~20wk	82,13	79,76
P6~40wk	92,06	94,29

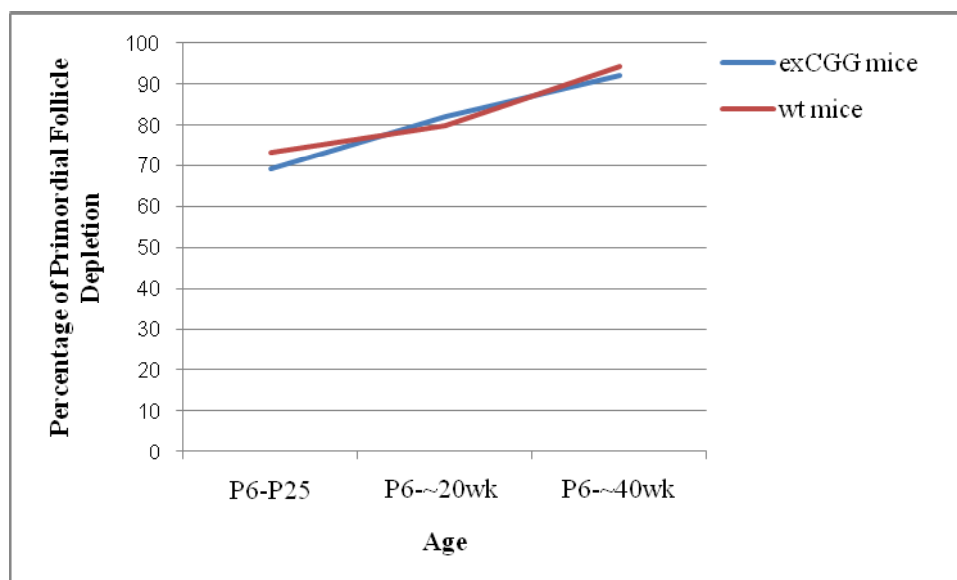


Figure 5.4. Percentage of primordial follicle depletion from P6 to varying age in premutation and wt mice. Primordial follicle from premutation mice and wt mice were progressively depleted during reproductive lifespan and the trend of primordial depletion from two premutation and wt mice are similar.

5.1.2. Recent corpora lutea identification in premutation mice

The presence of recent ovulations in ovaries from premutation mice were compared to wt mice using recent corpora lutea identification to identify the early cessation of ovulation cycle. Recent corpora lutea were detected by its appearance as a large rounded or irregularly shaped structure consists of lutein cells without oocyte in the middle.



Figure 5.5. Photomicrograph of ovarian histology of premutation mice. (A) showing recent corpora lutea determined by its appearance as a large rounded shaped structure without oocyte in the middle and stained homogeneously bright blue because it is contain of lutein cells. (B) showing old corpora lutea determined by its appearance as a large irregularly shaped structure stained homogeneously bright red because of its cellular elements degenerate and undergo autolysis and change with fibrous tissue. Magnification x 40.

There is no corpora lutea from P25 ovaries. Recent ovulation were showed only in 60% of 20-week-old premutation mice (n=10), whereas in wt mice recent ovulation were detected in all ovaries studied (n=6; 100%). There is no significance difference of these features between premutation mice and wt mice (p=0.234). (See table 5.3)

A total lack of recent corpora lutea was found in 40-week-old premutation mice (n=7), whereas in wt mice 78% (n=9) of ovaries contained recent corpora

lutea. Using Fischer exact test there is significance difference of these features between premutation mice and wt mice ($p=0.003$). (See table 5.4)

Table 5.3. Proportion of presence recent corpora lutea from ~20wk premutation mice compared to wt mice

Mice group	Recent corpus lutea		p value ^{*)}
	Yes	No	
premutation mice (n=10)	6 (60%)	4 (40%)	0,234
wt mice (n=6)	6 (100%)	0 (0%)	

*) Fischer Exact Test

Table 5.4. Proportion of presence recent corpora lutea from ~40wk premutation mice compared to wt mice

Mice group	Recent corpus lutea		p value ^{*)}
	Yes	No	
premutation mice (n=7)	0 (0%)	7 (100%)	0,003
wt (n=9)	7 (77,8%)	2 (22,2%)	

*) Fischer Exact Test

5.1.3. *Fmr1* mRNA levels

Fmr1 mRNA levels were analyzed in P25 ovaries from premutation mice using Reverse transcriptase and Quantitative PCR (RT and Q PCR) to support the hypothesis of RNA gain toxicity in POI. Using specific primer sets for *Fmr1* transition exon 5/6 and primer sets for internal controls (*Gapdh*), Ct values (cycle time to reach threshold of number cDNA) of samples were obtained from the machine. Fold change were obtained by subtracted Ct values of the reference gene from the Ct value of the target gene, which gives the Δ Ct. Δ Ct of wild type mice then subtracted from Δ Ct premutation mice, which is designated as the

$\Delta\Delta Ct$. $2^{-\Delta\Delta Ct}$ results in fold change. The fold change of *Fmr1* mRNA levels are depicted in Table 5.5.

Table 5.5. Fold change of *Fmr1* mRNA levels in P25 ovaries from premutation mice compared to wt mice at the same age.

Mice	Repeat length	Fold change vs wt
1	~137/~199	4x
2	~134/~173	3x
3	~134/~173	6x
4	~140/~140	3x
5	~144/~144	5x

Fmr1 mRNA levels were elevated 3-6 fold with an average of four-fold compared to age-matched wt mice.

5.2. Discussion

POI can occur at various stages of follicular development and could be related to 1) an initial decrease in the primordial follicle pool, 2) altered recruitment of the dormant follicle, 3) interrupted maturation of the follicle or 4) increased follicular atresia. FMRP, the protein product of the *FMRI* gene, is expressed in several tissues that may influence any of these development stages: in the brain, in the region that is critical for hormonal regulation, in the primordial germ cells in the fetus, and in the granulosa cells and oocyte of developing follicles in adults (2). Thus, histopathological studies in ovarium are important to understand POI.

Bachner et al (1993) demonstrated that *FMRI* serves a special function during germ cell proliferation in both males and females (41). Studies in

Drosophila showed that *dFMR1* null ovaries contain egg chambers with both fewer and supranumerary germ cells (42). FMRP also controls the level of *Cbl* mRNA in the ovary by reducing *Cbl* gene dosage (42). These results suggest that FMRP controls germline proliferation during oogenesis by regulating the expression of *Cbl* in the developing ovary. *Cbl* gene is responsible for germ cell proliferation. In *Drosophila*, *dFMR1* is also known to be involved in germ cells and oocyte specification (43).

Immunohistochemistry study demonstrated that in murine ovaries, *Fmrp* is predominantly expressed in granulosa cells and oocytes of developing follicles (39). Willemsen et al (2009) demonstrated that *Fmrp* expression in ovary of premutation mice is enhanced compared to wt mice.

Primordial follicles are formed just after birth in mice, and this pool of primordial follicles constitutes the complete supply of ovarian follicles during reproductive life (48). Initiation of primordial follicle growth occurs as soon as they are formed, but not all primordial follicles start to grow at the same time and some will remain dormant for months in rodents and even for years in the human . The factors that regulate initiation of primordial follicles growth are still largely unknown (18).

The effect of the fragile X premutation on primordial follicle formation was tested using ovaries from P6 mice. Ovaries of P6 mice provide a useful system for the study of factors that influence the primordial follicle growth, since they contain predominantly primordial follicles, and no growing follicles.

The absence of a reduced number of primordial follicles in P6 premutation mice suggests that the fragile X premutation has no effect on the initial pool of primordial follicles from birth. In fact, these results show a trend in the opposite direction, that is a higher number of primordial follicles in premutation mice. This finding seems to contradict with hypothesis that there is a reduction in the number of oocyte at birth in female carrying premutation allele (36).

An explanation for a trend showing higher number of primordial follicles in premutation mice is that Willemsen et al (unpublished, reviewed in Dept of Clinical Genetics ErasmusMC Rotterdam 2009) demonstrated the expression of *Fmrp* in ovaries of premutation mice is increased than wt mice. The increase expression of *Fmrp* in ovary of premutation mice might cause the *Cbl* mRNA levels lower than normal. *Cbl* gene is responsible for inhibition of germ cell proliferation (42). The reduction of *Cbl* levels more than normal results in germ cells over proliferation.

Following this results, an effect of the premutation on next stages of folliculogenesis were determined. The percentage of primordial follicle depletion from P6 to varying age in premutation mice, showing the lower number of primordial follicle depletion compared to wt mice suggest that in premutation mice there is an altered recruitment of the primordial follicles. We hypothesize that the altered initial recruitment in premutation mice will lead to reduced number of growing follicles at later stages. This is confirmed by the reduced ovulation at ~20wk and ~40wk premutation although there is higher initial pool of primordial follicles.

The underlying mechanism of this alteration in these mice has not yet been defined. A possible cause might be a RNA-gain of function-toxicity. Recently, a similar mechanism has been postulated for the elevated levels of *FMRI* mRNA in the development of an adult-onset neurological disorder (FXTAS) in males who carry the fragile X premutation.

Previous studies demonstrated that at the molecular level, all individuals with premutation alleles show elevated *FMRI* mRNA levels, 2-10 times higher than normal, in peripheral blood leukocytes, but normal or reduced FMRP levels (45). Using Q and RT PCR analysis using RNA isolated from mice brain, Willemsen et al (2003) showed a two fold increase of *Fmr1* mRNA levels at different ages in premutation mice, when compared with age-matched wt mice (20). Four-fold elevated *Fmr1* mRNA in ovaries of 25-day-old premutation mice in comparison with age-matched wt mice is reported here. Thus, these results confirm the earlier findings in brain tissue from the premutation mice (20). This finding does not mean that increase levels of *Fmr1* mRNA is always causing an altered initial recruitment of primordial follicles, because Tejada et al (2008) demonstrated that increased levels of *FMRI* mRNA was found in lymphocytes from a female carrying a premutation without POI. Actually, POI occurs only in 30% of female premutation carriers (66).

The unlinear relationship between *FMRI* repeat size and POI in female premutation carriers has been reported, and the risk of development of POI appears to increase with premutation repeat sizes between 59 and 99 CGG. Higher repeat sizes (>100 CGGs) decreases the risk of POI. Females carrying a

premutation repeat size between 80-100 have the highest risk to develop POI (13). Premutation mice that have CGG repeat sizes between 100-199 repeats were used, which does not fully reflect the highest risk group in the human situation. On the other hand, female carriers of the premutation are always heterozygous for the premutation allele, whereas our premutation mice were homozygous.

Despite these differences, it is concluded that the premutation does not cause depletion in the initial pool of primordial follicles from birth. In contrast, the pool of primordial follicles at later stages is higher in premutation mice compared to wt mice, and might be involved in mechanisms that may influence the initial recruitment of primordial follicles.