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Commentary: FMRpolyG accumulates in mural granulosa cells of *FMR1* premutation carriers and in premutation transfected COV434 cells

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1. Abbreviations

FXPOI: fragile X-Associated Premature Ovarian Insufficiency

FXTAS: fragile X-associated tremor/ataxia syndrome

RAN translation**:** repeat associated non-AUG initiated translation

FMR1: fragile X mental retardation 1

FXS: Fragile X Syndrome

COH: controlled ovarian hyperstimulation

FMR1 premutation status is defined in the presence of an expanded CGG tract (n=55-200). *FMR1* premutation is associated with increased risk of fragile X-associated premature ovarian insufficiency (FXPOI) in females [1,2], and fragile X-associated tremor/ataxia syndrome (FXTAS) predominantly in males [3,4]. FXPOI is clinically significant as while in the general population only 1% may suffer from premature ovarian insufficiency (POI), female *FMR1* premutation carriers are at a higher risk and about 20-25% may suffer declined fertility at a young age [5]. Symptoms of FXPOI include menopause before the age of 40 years and decreased fertility as evident by age matched abnormal ovarian reserve biomarkers. Moreover, some *FMR1* premutation carriers might exhibit a reduced ovarian response to controlled ovarian hyperstimulation (COH), where despite the administration of high gonadotropin dosages, embryo yield remains low [5-11].

The pathobiology of FXPOI is still obscure, whereas the knowledge regarding the molecular mechanism of FXTAS is accumulating. The RNA gain-of-function mechanism resulting in RNA toxicity and a non-canonical protein translation creating a cryptic polyglycine-containing protein, called FMRpolyG, are two major suggested mechanisms of FXTAS well described in literature [12,13]. FMRpolyG formation is triggered by the expended CGG repeats through a mechanism named repeat associated non-AUG initiated (RAN) translation. A recent study published by Rodriguez, C. M. et al [14] identified and defined a native physiological

purpose for CGG repeats and RAN translation at the *FMR1* locus. They proved that CGG RAN translation acts as a regulatory upstream ORF that restricts the production of FMRP in neurons.

Former studies have demonstrated FMRpolyG accumulation in ubiquitin-positive inclusions in Drosophila, cell culture, mouse disease models [13,15,16], as well as in FXTAS patient brains which may lead to protein mediated neurodegeneration [4,17]. A toxic effect of FMRpolyG accumulation was demonstrated by Sellier and Todd et al. in FXTAS drosophila [15] and in transgenic mice [13] models. Of notice, inclusion formation, motor dysfunctions, and reduced lifespan was observed in transgenic mice producing both CGG RNA repeats and the poly (glycine) protein (99xCGG with FMRpolyG expression), but not in animals expressing only enlarged CGG repeats (99xCGG without FMRpolyG mouse) [13]. Xuan Phuoc Nguyen et al. detected FMRpolyG expression in peripheral blood monocyte cells (PBMCs) from *FMR1* premutation carriers [18]. These findings suggest that the translation of expanded CGG repeats into FMRpolyG may have a main role in FXTAS and FXPOI pathophysiology [9]. Studies investigating the mechanism of FXPOI are scarce. Buijsen et al. investigated FMRpolyG expression in the ovary and demonstrated its expression in extra-follicular ovarian cells of a 42 years old FXPOI patient, however not in folliculogenesis related cells [20]. Therefore, the main aim of our study was to demonstrate FMRpolyG accumulation in mural granulosa cells of *FMR1* premutation carriers. Detecting FMRpolyG accumulation in folliculogenesis related cells is crucial to elucidate FXPOI pathogenesis.

In contrary to previous studies which showed a single large ubiquitin-positive nuclear inclusion body in the nervous system and in numerous systemic organs [15,21], our study revealed FMRpolyG accumulation mainly in the cytoplasm of mural granulosa cells rather than in the nucleus. Moreover, FMRpolyG accumulation and its co-localization with ubiquitin varied within the group of the *FMR1* premutation carriers; we observed multiple small FMRpolyG granules scattered in the cytoplasm while a large portion did not co-localize with ubiquitin staining. We postulated that FMRpolyG accumulates mainly in the cytoplasm and less in the nucleus due to the different characterizations and functions of mural granulosa cells compared to brain cells. Ubiquitin-positive FMRpolyG containing an intranuclear inclusion were shown in non-dividing or slow dividing cells [22,23], whereas in our study FMRpolyG accumulated mainly in the cytoplasm of granulosa cells, representing rapidly dividing cells. Studies on rapidly dividing cells showed higher proteasome activity, disposing toxic proteins during cell division[24], while in slowly growing cells proteasome subunits have been downregulated [25]. Furthermore, the accumulation of nuclear aggregates of FMRpolyG is a continuous process. We propose to further investigate whether the proteasome complex disposes of accumulated FMRpolyG during cell division. In this study, we report observations demonstrating the accumulation of FMRpolyG. Nonetheless, our findings cannot explain the variations in FMRpolyG aggregates formation between FXTAS and FXPOI and additional studies are essential.

A second purpose of this study concerns the importance of establishing a well representative cell line disease model to investigate the effect of RNA toxicity and RAN translation in folliculogenesis related cells. Isolating the contribution of the RNA-gain-of-function mechanism from RAN translation mechanism's effect is crucial. To accomplish that, we transfected human ovarian granulosa cell tumor, COV434 with two plasmids both expressing 99CGG repeats, but only one enables FMRpolyG expression [19], as should naturally occur in *FMR1* premutation carriers. FMRpolyG aggregates were found only in COV434 transfected with expended CGG repeats and the ability to express FMRpolyG. We suggest that the transfected COV434 cells might be a suitable model to investigate *FMR1* premutation folliculogenesis related cells and might shed insight on the function of each mechanism in FXPOI pathogenesis.

Detecting FMRpolyG accumulation in folliculogenesis related cells supports previous observations, and implies a possible common protein-mediated toxic mechanism for both FXPOI and FXTAS. Nonetheless, further research is imperative to properly comprehend the RAN translation mechanisms as well as its role in *FMR1* premutation carriers and FXPOI pathogenesis.

2. Declarations

Ethics approval and consent to participate

The original study was approved by the Institutional Ethical Review Board of Sheba Medical Center, Israel. All patients that were included in this study signed a written informed consent. Helsinki no. 8707-11-SMC and 6140-19-SMC.

3. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

4. Competing interests

The authors declare that they have no competing interests.

5. Funding

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6. Authors Contribution

MFG designed and conducted the study, analyzed the results, wrote and approved the final manuscript. LMH reviewed and approved the final manuscript. RO and YC provided oversight and assisted in manuscript editing and critical review. All authors read and approved the final manuscript.

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