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X-linked *miR-506* family miRNAs promote FMRP expression in mouse spermatogonia

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See also: **M Ramaiah *et al*** (February 2019)
See reply: **M Ramaiah *et al***

MicroRNAs (miRNAs) are ~22 nt small RNAs that function to regulate gene expression at post-transcriptional levels [1]. ~10% of testicular miRNAs are transcribed from several large miRNA clusters on the X chromosome, and most of these X-linked miRNAs are preferentially expressed in the testis [2,3]. For example, the *miR-506* family contains 21 miRNAs that are transcribed from five large miRNA clusters encompassing a ~62 kb region near *Slitrk2* and one large miRNA cluster expanding ~22 kb region close to *Fmr1* on the X chromosome [4] (Fig 1A, Table EV1). Although *miR-506* family miRNAs have undergone rapid evolution, the two protein-coding genes flanking these miRNA clusters, i.e., *Fmr1* and *Slitrk2*, remain highly conserved across all mammalian species [5,6]. *Fmr1* and *Slitrk2* are known to be involved in human fragile X mental retardation [7] and Tourette's syndrome [8], respectively. The close proximity between the *miR-506* family and *Fmr1* has prompted a hypothesis that *Fmr1* might be a target of the *miR-506* family. A recent paper by Ramaiah *et al* reports that the *miR-506* family miRNAs, which they termed *Fx-mir* miRNAs, function to repress *Fmr1* (encoding FMRP) expression in Sertoli cells based on expression profiling analyses and luciferase-based reporter assays *in vitro* [5]. Our laboratory first reported the exclusive or preferential expression of 77 X-linked miRNAs in the murine testes [3], and we happen to have generated knockout (KO) mice lacking the five miRNA clusters encoding 18 out of 21 *miR-506* family miRNAs. Major findings reported by Ramaiah *et al*, including Sertoli cell expression of the *miR-506* family miRNAs and their repressive effects on *Fmr1*, appear to be inconsistent with either published or our own data.

The authors claim that the X-linked miRNAs are mainly expressed in Sertoli cells based on three lines of evidence. First, qPCR analyses of *miR-506* family miRNAs were performed using Sertoli cells and interstitial cells purified from adult mouse testes as well as germ cells of unknown sources with unknown purity. It is well known that the purity of each of the testicular cell types can vary a lot due to batch effects. The authors also showed that the highest expression was detected in Sertoli cells, but this result is not consistent with any of the data previously reported by multiple independent groups using different methodologies including qPCR [3], RNA-FISH [9,10], and RNA-seq [6,11], all showing that these miRNAs are predominantly expressed in germ cells, especially in spermatogonia [6,9–11], and that levels of these miRNAs are extremely low in Sertoli cells [11]. Using Sertoli cells and spermatogonia (both type A and type B) purified from CD-1 mice at postnatal day 8 (P8), we conducted small RNA sequencing (sRNA-seq) analyses (Appendix Supplementary Methods and Appendix Table S1) (Fig 1B). Our sRNA-seq data also indicated that abundance of the majority of the *miR-506* family miRNAs was much higher in spermatogonia than in Sertoli cells (Fig 1B). Although the STA-PUT method that we used led to relatively high purity (> 85% for both Sertoli cells and spermatogonia), minor cross-contamination would have biased the data given the superb sensitivity of PCR. Moreover, Sertoli cells and spermatogonia in immature testes (at P8) may have different expression profiles compared with those in adult testes. Therefore, our data remain inconclusive, but at least consistent with the literature in general. An ideal way to clarify this controversy would be single-cell co-sequencing of miRNAs and mRNAs [12], in which mRNAs could be used as cell type markers and relative abundance of miRNAs can be more accurately determined. Second, the authors demonstrated that the *miR-506* family miRNAs showed elevated levels in germ cell-deficient testes of mice (*jsd/jsd* mice) and humans (Sertoli cell-only (SCO) patients) compared with normal controls. However, the testes of *jsd/jsd* mice are known to contain some spermatogonia, albeit in a small number [13], and SCO human testes may also contain some germ cells, as acknowledged by the authors as well [5]. Therefore, qPCR-based detection of the *miR-506* family miRNAs in these so-called germ cell-deficient testes cannot exclude their expression in spermatogonia. Third, the authors ascribed the decreased expression of some of these miRNAs after P15 to the cessation of Sertoli cell proliferation. However, in P15 testes, the number of spermatocytes, especially mid- to late pachytene spermatocytes, is increasing drastically [14,15]. The significantly decreased levels of these miRNAs may well be due to the dilution effects of the increasing number of spermatocytes. Taken together, the notion that the *miR-506* family miRNAs are mainly expressed in Sertoli cells needs to be better supported with further experiments.

The conclusion that the *miR-506* family miRNAs repress *Fmr1* in Sertoli cells was

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based on Western blots and luciferase-based reporter assays *in vitro*. The authors showed that overexpression of some of the *miR-506* family miRNAs (except *miR-883a*) repressed both *Fmr1* luciferase activity and protein levels in a P19 murine teratocarcinoma cell line and an immortalized Sertoli cell line MSC1, whereas knock-down of these miRNAs increased *Fmr1* luciferase activity

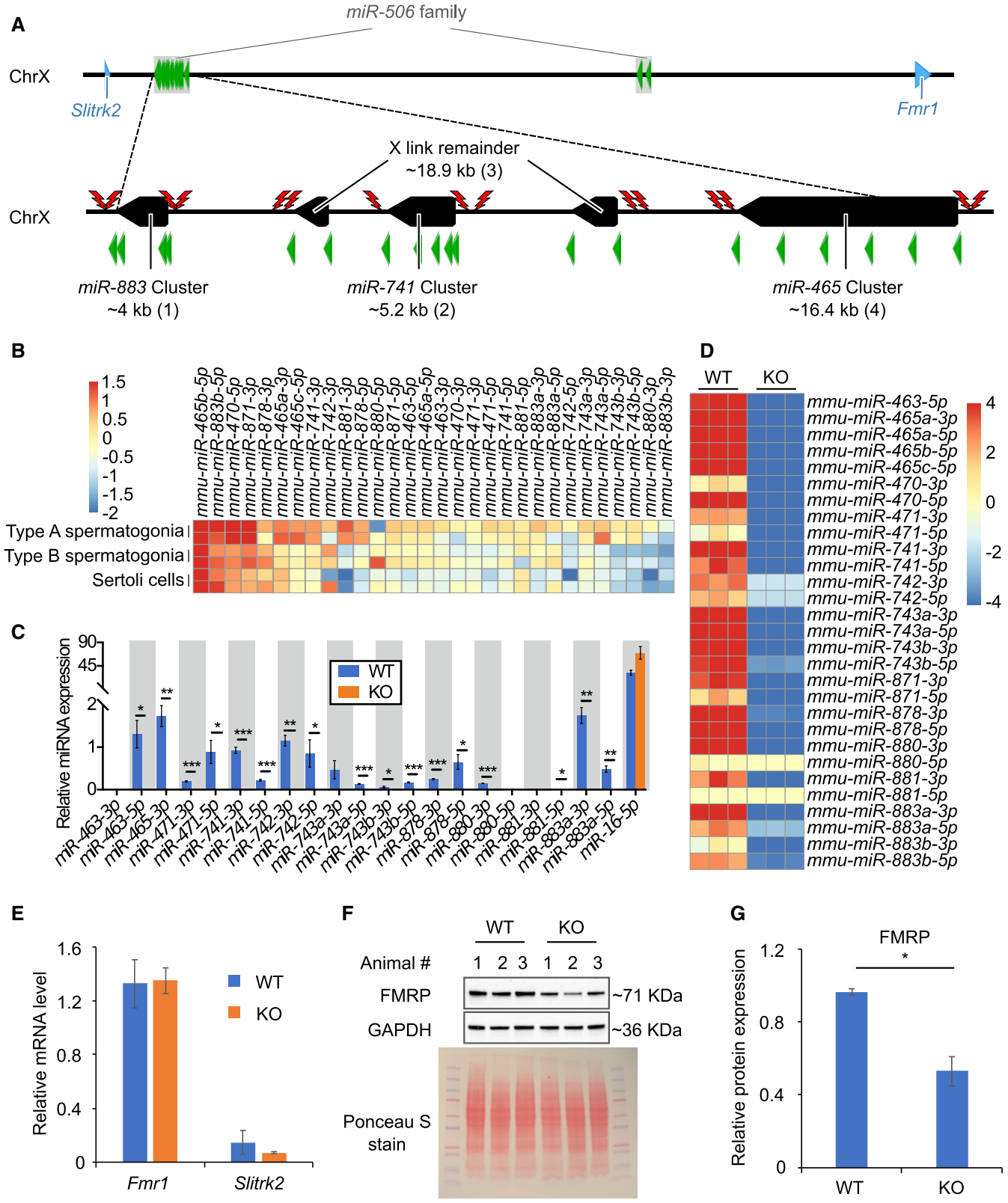

Figure 1.

Figure 1. X-linked *miR-506* family miRNAs promote FMRP expression in mouse spermatogonia.

(A) Schematic illustration of the strategy used for generation of the whole *miR-506* miRNA cluster KO mice using CRISPR-Cas9. The red lightning bolt represents the gRNAs used, and its right and left orientations indicate the gRNAs targeting the reverse and forward strands of the genomic DNA, respectively. Green and black arrows indicate precursor miRNAs and miRNA clusters, respectively. The whole cluster KO was achieved through sequential deletion of individual clusters, and the number inside the parenthesis shows the order of CRISPR-Cas9-based deletion. (B) Heatmap showing the abundance of the *miR-506* family miRNAs in spermatogonia and Sertoli cells based on sRNA-seq. Two replicates were done for each cell type. (C) TaqMan probe-based qPCR analyses of the *miR-506* family miRNAs in WT and KO adult testes. *miR-16-5p* was used as a loading control. Expression levels were normalized to *U6*. Data are presented as means ± SEM, $n = 3$. (D) Heatmap of small RNA-seq data showing expression levels of the *miR-506* family miRNAs in WT and KO adult testes. Testes of biological triplicates were used for small RNA-seq analyses. (E) qPCR analyses of *Fmr1* and *Slitrk2* mRNA levels in WT and KO adult testes. Expression levels were normalized to *Gapdh*. Data are presented as means ± SEM, $n = 3$. (F) A representative image of Western blots of FMRP and GAPDH in WT and KO testes. Upper panel, blots of FMRP and GAPDH proteins; lower panel, Ponceau S staining of the membrane. (G) Quantitative analysis of FMRP levels based on Western blot results. The density of the bands was analyzed using ImageJ, and FMRP levels were normalized to GAPDH. Data are presented as means ± SEM, $n = 3$. *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

in a spermatogonial stem cell line called GS. However, those miRNAs and *Fmr1* have to be expressed in the same cell type if such a miRNA-mRNA targeting relationship exists physiologically. Two papers that the authors cited as the evidence for FMRP (encoded by *Fmr1*) expression in Sertoli cells actually reported that FMRP was mainly expressed in spermatogonia and hardly detectable in the Sertoli cells in either human [16] or mouse [17] testes. Another independent study has also demonstrated that FMRP is predominantly expressed in spermatogonia in human testes [18]. We examined the datasets from two recent single-cell RNA-seq studies on both human and mouse testes [19,20], and found that *Fmr1* mRNA is exclusively expressed in germ cells, especially in spermatogonia (Table EV2). So, if those miRNAs are expressed in Sertoli cells, how can they target *Fmr1* in spermatogonia?

To reveal the function of the *miR-506* family miRNAs, we generated KO mice lacking the five large miRNA clusters encoding 18 out of 21 of the *miR-506* family miRNAs using CRISPR-Cas9 (Fig 1A, Table EV1) as previously described (Appendix Supplementary Methods and Appendix Table S1) [15,21]. Lack of expression of the *miR-506* family miRNAs was validated using the TaqMan probe-based qPCR (Fig 1C) and small RNA-seq (Fig 1D) (Appendix Supplementary Methods and Appendix Table S1). The KO mice developed normally and showed no discernible changes in testicular histology. Although qPCR analyses detected no significant changes in *Fmr1* and *Slitrk2* mRNA levels between WT and KO testes (Fig 1E), Western blots revealed that FMRP levels were significantly down-regulated in the KO testes. Therefore, these miRNAs do not repress, but rather promote FMRP expression (Fig 1F and G) probably through enhancing translational efficiency without affecting mRNA stability *in vivo*.

In summary, both published and our own data suggest that Sertoli cells may not be the major site of expression for *Fmr1*/FMRP and the *miR-506* family miRNAs, and these miRNAs, instead of repressing, actually promote FMRP expression under physiological conditions.

Data availability

The small RNA-seq datasets have been deposited into the SRA database with accession no.: PRJNA558973 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA558973). The modeling computer scripts can be found on GitHub (https://github.com/biogramming/AASRA).

Expanded View for this article is available online.

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Author contributions

ZW and WY conceived and designed the research. ZW, YX, YW, DM, SW, DO, SY, KZ, SB, and HZ. performed bench experiments. ZW and YX analyzed the data. ZW and WY wrote the manuscript. All reviewed and agreed with the contents of the manuscript.

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