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CORRESPONDENCE

Maternally expressed miR-379/miR-544 cluster is dispensable for testicular development and spermatogenesis in mice

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MicroRNAs (miRNAs) are endogenously expressed, ~19–23 nt non-coding RNAs (ncRNAs) that regulate gene expression at the post-transcriptional level, closely related to spermatogenesis and male infertility (Bouhallier et al., 2010; Comazzetto et al., 2014; Yuan et al., 2015). The *miR-379/miR-544* locus is the largest known placental mammal-specific miRNA cluster, whose 24 miRNA genes are expressed only from the maternal allele, and are located within the highly conserved imprinted *Dlk1–Dio3* region (Figure 1a). A correct dosage of imprinted genes encoded at the mouse *Dlk1–Dio3* genomic interval is essential for embryonic growth and postnatal survival, as well as for muscle growth, skeletal, and neuronal development (Seitz et al., 2004). A cluster of imprinted miRNAs encoded by imprinted *Dlk1–Dio3* locus were reported to be high expressed in ES cells and 4n-iPS cells and correlated with the pluripotency levels of mouse stem cells (Liu et al., 2010). The primary transcript of *miR-379/miR-544* cluster was enriched in embryonic and perinatal skeletal muscle, and is associated with callipyge phenotype in young animals (Gao et al., 2015). However, whether the *miR-379/miR-544* cluster at imprinted *Dlk1–Dio3* region contributes to testicular development and spermatogenesis remains elusive. We examined expression levels of the *miR-379/miR-544* precursor transcript containing all 24 miRNAs in nine different adult mouse organs and embryonic day 17.5 (E17.5) mouse testes using semi-quantitative RT-PCR and quantitative RT-PCR (RT-qPCR). Interestingly, we found that the primary transcript of the *miR-379/miR-544* cluster was only expressed in the brain and E17.5 testis (Figures 1b and 1c), suggesting *miR-379/miR-544* may function in brain and testicular development. We then further measured the primary transcript levels of the *miR-379/miR-544* cluster in postnatal developing testes and observed the *miR-379/miR-544* cluster primary transcripts displayed a gradually declining expression pattern from postnatal 0 (P0) to P42 testis, and the highest expression levels were detected at P0 and lowest detectable expression was observed at P28 testis (Figure 1d–e). This dynamic expression pattern in testis was similar to that reported for the *miR-379/miR-544* cluster in skeletal muscle (Gao et al., 2015). Given that, we hypothesize that the *miR-379/miR-544* cluster may be involved in the first wave of spermatogenesis and testicular development.

To investigate the physiological role of the *miR-379/miR-544* cluster in vivo, we established a knockout (KO) mouse line carrying three *loxp* sites. Following by crossed with *Stra8-Cre* and/or *Zp3-Cre* mouse lines, and a ~9.4-kb genomic DNA sequence deletion was created in male or female gametes, thus the paternal or maternal *miR-379/miR-544* cluster null allele was generated (Figure 1f). Because the *miR-379/miR-544* cluster is located in an imprinting region where only the maternally inherited allele is competent for transcription (Seitz et al., 2004), we generated two types of genetically comparable heterozygotes from two reciprocal parental crosses (Figure 1g): (1) wild-type (WT) females with *Stra8-Cre;miR-379/544*$^{+/lox}$ males and (2) WT males with *Zp3-Cre;miR-379/544*$^{+/lox}$ females. In principle, only

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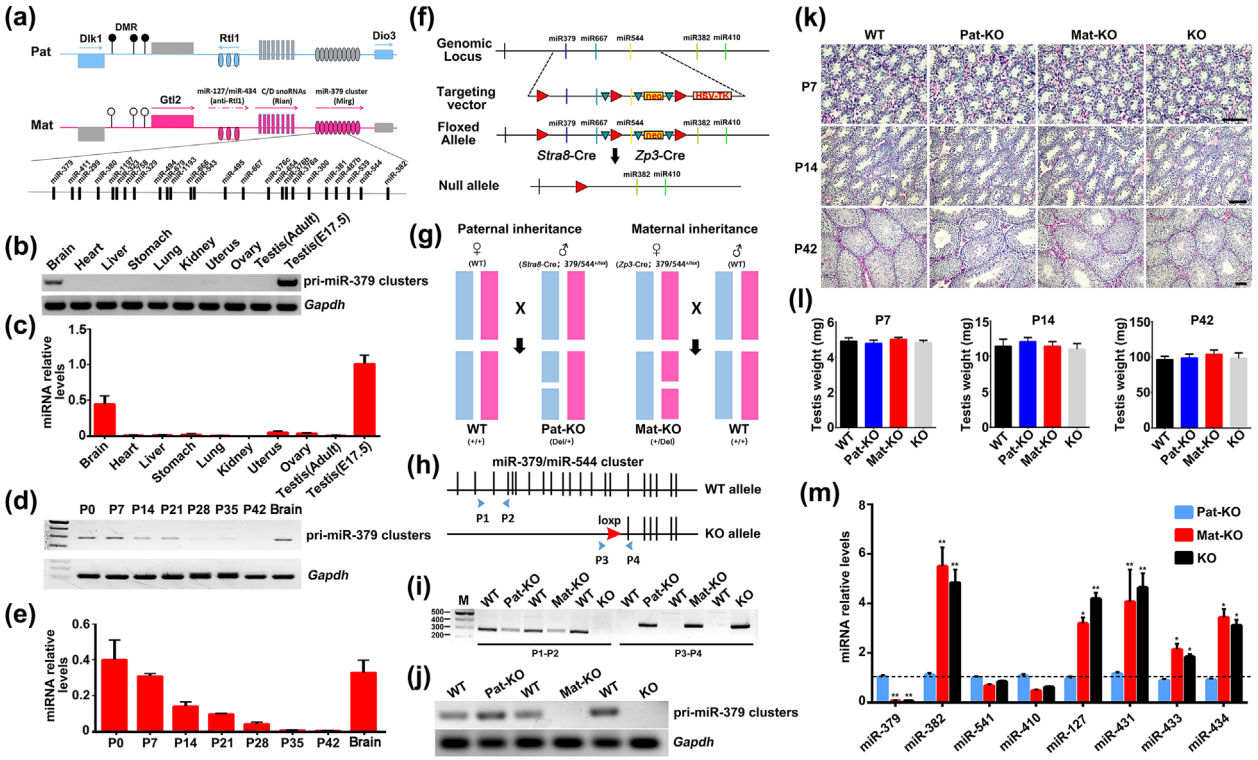

FIGURE 1 Imprinted *miRNA-379/miR-544* cluster is dispensable for spermatogenesis. (a) Schematic representation of the imprinted *Dlk1–Dio3* locus on the mouse distal chromosome 12. Paternally expressed protein-coding genes (*Dlk1*, *Rtl1*, and *Dio3*) are symbolized by blue rectangles, while maternally expressed *miR-379* cluster and C/D snoRNA genes are depicted by pink ovals and rectangles, respectively. Silent alleles are colored in gray. Note that numerous miRNAs and C/D snoRNAs are embedded within and processed from introns of non-coding gene transcripts, namely *Gtl2* (pink rectangle box), *Anti-Rtl1*, *Rian*, and *Mirg*. Differentially methylated regions (DMR) are indicated by filled and open lollipops (methylated and unmethylated, respectively). The relative positions of *miR-379/miR-544* cluster are indicated in the enlarged inset. Mat, maternal chromosome; Pat, paternal chromosome. (b and c) Semi quantitative RT-PCR and quantitative PCR analyses of pri-miR-379/miR-544 cluster transcript expression levels in nine different adult organs and embryonic 17.5 testes. (d and e) Semi-quantitative RT-PCR and quantitative PCR analyses of pri-*miR-379/miR-544* transcript cluster expression levels in developing testes and brain (as positive control). Testes at postnatal Day 0 (P0, newborn), P7, P14, P21, P28, P35, and P42 were analyzed. *Gapdh* as the loading control in b and d, data are normalized with *Gapdh* and shown graphically as mean ± SEM of three independent samples in c and e. (f) Schematic representation of the *miR-379/miR-544* floxed strategy. Three loxp sites were inserted into the genomic DNA. The first loxp site was targeted upstream of *miR-379*, the Frt-loxp cassette was inserted downstream of *miR-667* and the third loxp site with a PGK-Neo cassette was introduced downstream of *miR-544*. Mice containing the floxed allele were crossed with *Stra8-Cre* and/or *Zp3-Cre* mouse lines to generate the *miR-379/miR-544* cluster (contain 24 miRNAs) paternal or maternal null allele. (g) A schematic representation of the two parental crosses performed to generate Pat-KO and Mat-KO individuals and their WT littermates using *Cre-loxp* breeding system. *Stra8-Cre;miR-379/544*$^{+/lop}$ males can produce null allele sperm and *Zp3-Cre;miR-379/544*$^{+/lop}$ females can produce null allele oocytes. Paternally and maternally inherited chromosomes are symbolized by blue and pink rectangles, respectively. (h and i) Genotyping analyses of WT mice, heterozygotes (Pat-KO and Mat-KO), and homozygotes (KO). The P1–P2 primer pair was used to detect the WT allele, whereas the P3–P4 primer pair was used for the *miR-379/miR-544* cluster null allele. M, DNA maker (bp). (j) Semi-quantitative RT-PCR analyses of pri-miR-379/miR-544 cluster transcript expression levels in WT, Pat-KO, Mat-KO and KO mouse testes at postnatal Day 7 (P7). *Gapdh* as the loading control. (k) The representative images of Periodic acid-Schiff-stained WT, Pat-KO, Mat-KO, and KO mouse testes section at different postnatal days (P7, P14, and P42 were examined). Scale bar = 100µm. (l) Histogram showing the testis weights of WT, Pat-KO, Mat-KO, and KO mice are all comparable at age of postnatal day 7, 14, and 42. Data are shown as mean ± SEM of three independent samples. (m) qRT-PCR analyses of selected individual miRNA scattered around *miR-379/miR-544* cluster in WT, Pat-KO, Mat-KO, and KO testes at P7. Data were normalized with U6 snRNA and shown graphically as mean ± SEM. Expression levels of WT were arbitrarily set to 1. **p*< 0.05, ***p*< 0.01 compared to WT (*n* = 3 in each of genotype, Student's *t*-test)

offspring having inherited the deletion allele from a *Zp3-Cre;miR-379/544*$^{+/lox}$ mother (here called Mat-KO) are lacking *miR-379/miR-544* expression. In contrast, offspring having inherited the deletion allele from a *Stra8-Cre;miR-379/544*$^{+/lox}$ father (here called Pat-KO) express the miRNA cluster normally. The genotypes of the mutant mice (Mat-KO or Pat-KO) or homozygous deletion (KO) mice were confirmed via PCR analyses (Figures 1h and 1i). Moreover, the expression levels of the primary transcript of the *miR-379/miR-544* cluster in Mat-KO, Pat-KO, and KO in P7 testes were further confirmed by RT-PCR analysis. The analyses showed that Pat-KO testis expressed a comparable level of the *miR-379/miR-544* cluster transcripts to WT testis, whereas Mat-KO and KO testis did not (Figure 1j). The Mat-KO, Pat-KO, and KO

pups were born in the expected Mendelian ratios and were viable, and did not exhibit any gross physical abnormalities (data not shown).

To determine the fertility of the *miR-379/miR-544* cluster Mat-KO and KO males, we performed fecundity tests using Mat-KO, Pat-KO, and KO adult males bred with fertility-proven adult WT females. Our breeding data revealed no significant difference in either litter size or litter interval compared to WT breeding pairs (data not shown), suggesting that the *miR-379/miR-544* cluster-null males (both Mat-KO and KO) are completely fertile. To examine the first wave of spermatogenesis and neonatal testis development in Mat-KO and KO animals, we did PAS staining for visualizing testicular seminiferous tubules at P7, P14, and P42. Compared to WT littermates, both Mat-KO and KO males displayed normal testicular histology and testis weight (Figures 1k and 1l), suggesting that the first wave of spermatogenesis and neonatal testicular development were normal. Taken together, our data indicate that *miR-379/miR-544* cluster is not required for spermatogenesis and testicular development in mice.

We analyzed the transcript levels of the neighbor maternal imprinting small ncRNAs within the imprinted *Dlk1–Dio3* region among WT, Pat-KO, Mat-KO, and KO neonatal testes (at P7) by RT-qPCR. We found that the expression levels of *miR-382*, *miR-127*, *miR-431*, *miR-433*, and *miR-434* were significantly increased in both M-KO and KO testes compared to those of WT and Pat-KO (Figure 1m). This suggests that the neighbor maternal imprinting small non-coding RNAs may have compensated for the loss of the *miR-379/miR-544* cluster functions, thus maintaining a normal phenotype in the *miR-379/miR-544* cluster deficient male testes. In summary, our findings demonstrate that the maternally expressed *miR-379/miR-544* cluster is not required for both testicular development and spermatogenesis in mice.

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