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MiR-125b-2 Knockout in Testis Are Associated with Targeting to PAP Gene, Mitochondrial Copy Number and Impaired Sperm Quality

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Abstract: microRNAs can cause male infertility by impacting sperm quality and impaired spermatogenesis. Since the miR-125 family plays an important role in regulating embryo development, but the function of miR-125b-2 in male reproduction remains unknown. In this study, we prepared a model of miR-125b knockout (KO) mice. Among the KO mice, the progeny test showed that litter sizes decreased significantly and the rate of non-parous females increased significantly ($p<0.05$). At the same time, the testosterone concentration increased significantly ($p<0.01$), with the remarkable decrease for estradiol ($p<0.05$). Moreover, sperm count decreased obviously ($p<0.05$) and the percentage of abnormal sperms increased significantly ($p<0.01$). Testicular transcriptome sequencing demonstrated that there were 173 up-regulated genes, including Papolb (PAP), and 151 down-regulated genes in KO mice compared with wild type (WT). KEGG and GO analysis showed many of these genes were

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involved in sperm mitochondrial metabolism and other cellular biological processes. Meanwhile, the sperm mitochondria DNA (mtDNA) copy number was increased significantly ($p<0.01$) in KO mice, but the integrity of mtDNA and nuclear DNA (nDNA) had no change. In the top 10 up-regulated genes, as a testis specific expressing gene, PAP can affect the process of spermatogenesis. Western blotting and Luciferase Assay validated that PAP was the target of miR-125b-5p. Intriguingly, we also found that both miR-125b and PAP were only highly expressed in germ cells (GC) instead of Leydig cells (LC) and Sertoli cells (SC), and miR-125b-5p could target PAP to regulate TM3 cell secretion of testosterone ($p<0.05$). Our study firstly demonstrated that miR-125b-2 could regulate testosterone secretion by directly targeting PAP and increase sperm mtDNA copy number to affect semen quality. The study indicated that miR-125b-2 had a positive influence on the reproductive performance of animal and could be a potential therapeutic target for male infertility.

Keywords: miR-125b-2; testis; PAP; reproduction; sperm; mitochondria

1. Introduction

Infertility is a common human health problem[1] and 40%–50% of all cases are due to male deficiency. In recent years, several clinical studies[2,

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3] have shown that sperm quality and impaired spermatogenesis can cause male infertility. The process of spermatogenesis is highly sensitive to fluctuations in the environment and involves numerous endocrine and paracrine signals to coordinate the self-renewal and differentiation of spermatogonial stem cells[4]. Appropriate levels of reproductive hormones such as testosterone are a key microenvironment for spermatogenesis[5]. Hormone screening can help define whether the male infertility is due to gonadotropin deficiency. Spermatogenesis is regulated by the expressions of thousands of genes. Molecular and cellular integrity of sperm cells are important for fertilization, and inappropriate gene expressions cause disorders in spermatogenesis and fertility[6, 7].

A recent study in various eukaryotes has shown that small RNA molecules, including small interfering RNAs (siRNAs) and microRNAs (miRNAs) have emerged as important regulators of gene expressions at the post-transcription or translation levels[3]. Several miRNAs are expressed abundantly in male germ cells, either throughout or during specific stages of spermatogenesis[8]. MiRNA is a class of highly conserved small noncoding RNAs that primarily bind to complementary sequences in the 3'- untranslated region (UTR) of their target mRNAs; this binding results in mRNA degradation and/or repression of mRNA translation[9]. The gonadotrope specific deletion of the dicer, an endoribonuclease involved in the biogenesis of miRNAs, leads to male

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and female infertility by completely abolishing the synthesis of the two gonadotropins[10, 11]. Mature miR-125b originates from two precursors: miR-125b-1 and miR-125b-2. There are many reports on miR-125b. It has been reported that miR-125b suppresses epithelial ovarian cancer cell migration and invasion[12], regulate p53 in embryonic stem cells[13], and has an important position in lipogenesis[14] and so on. And studies have also shown that the miR-125 family plays a crucial role in regulating zygotic genome activation in oocytes and embryos[15]. Interestingly, no information has been published on miR-125b-2 functions in male fertility.

Previous study found that all miR-125b-2 gene KO mice appeared dysgenesis. This suggests that miR-125b-2 might be associated with animal reproduction. This study used miR-125b-2 knockout mice model to study the mechanism of miR-125-2 in reproductive performance in order to provide useful drug targets and markers for infertility treatment.

2. Result

2.1. Phenotype of KO mice

The KO mice were verified by PCR using tail DNA with primers 5′-ACATTACTGTAAGTTCTGATCTATA-3′ and 5′-GTACCGATTCTGAAGATTGTAT-3′. The sequences of WT and KO mice were blasted (Figure 1). The seed sequence of miR125b-2 is CCCTGAGACCCTAACTTGTGAGGTATTTTAGTAACATCACAAG.

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Through statistical analysis, we found both WT and KO male mice grew normally and had no difference in body weight, testis size (Table 1). The in vivo data suggested that there was no difference in thephenotype when miR-125b-2 was knocked out in mice.

Figure 1. The sequences of WT mice and miR-125b-2 knockout homozygous mice were compared by bio XM software.

Group	BW(g)	TW(mg)
WT	29.12±0.78	166.50±8.43
KO	28.09±0.89	167.50±6.38

Table 1. Body and Testis weight (mean±SEM)*Significant difference ($p<0.05$). Body and testicular tissues of WT and KO mice were weighted (n=8). BW, body weight; TW, testis weight. n=8.

2.2. miR-125b-2 Knockout Causes Infertility Phenotypes

The effect and mechanism of miR-125b-2 on mouse reproductive performance was investigated. Male fertility was tested through four mating combinations, which included **WT♀ X KO♂, KO♀ X KO♂, WT ♂ X KO♀** and **WT♀ X WT♂**. Comparing WT♂ X KO♀ with WT♀ X WT♂, the litter size significantly reduced ($p<0.05$) and the percentage of non-parous females rose to 18% from 10%. Comparing WT♀ X KO♂

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with WT♀ X WT♂, the litter size was not significantly different (p>0.05) and the percentage of non-parous females rose to 27% from 10%. But when we compared KO♀ X KO♂ with WT♀ X WT♂, the litter size decreased significantly（p<0.01）and the percentage of non-parous females rose to 38% from 10% (Figure 2A-B). For a further exploration, we examined the mice's sperm and found that the sperm count decreased in KO mice (p<0.05) compared to the WT group, as shown in Figure 2C. There was also a marked increase (p<0.01) in percentage of abnormal sperms in miR125b-2 knockout mice (Figure 2D). This result suggested that miR125b-2 could reduce sperm quality and affect mouse fertility. To determine whether the reproductive hormone concentrations in the serum have changed or not between WT and KO mice, we examined the levels of T and E_2 in the WT and KO serum. The WT group displayed an increase in T concentration (p<0.01) and a decrease in E_2 concentration (p<0.05) compared with KO group ((Figure 2E-F). The T and E_2 were secreted by Sertoli cells (SC) and Leydig cell (LC) of the testis. But comparative histological analysis of the testis sections revealed normal tubular architecture and size in KO mice, also showing no significant difference in LC and SC cells compared with WT mice (Figure 2G). T can be converted to E_2 under the enzymatic actions of P450. When the enzyme activity is suppressed, T cannot be effectively transformed into E2, which

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leads to focal hyperandrogenism[16]. Thus, to explain the mechanism of hormone changes, we quantified testicular P450 expression. As expected, KO displayed lower P450 mRNA expression than WT (Figure 2H). At the same time, we quantified hormone AR expression and KO exhibited no difference in AR mRNA expression compared with WT, but the relative level of AR protein is increased significantly in KO mice (p<0.01) (Figure 2I). It suggested that miR-125b-2 would affect the levels of sex hormones.

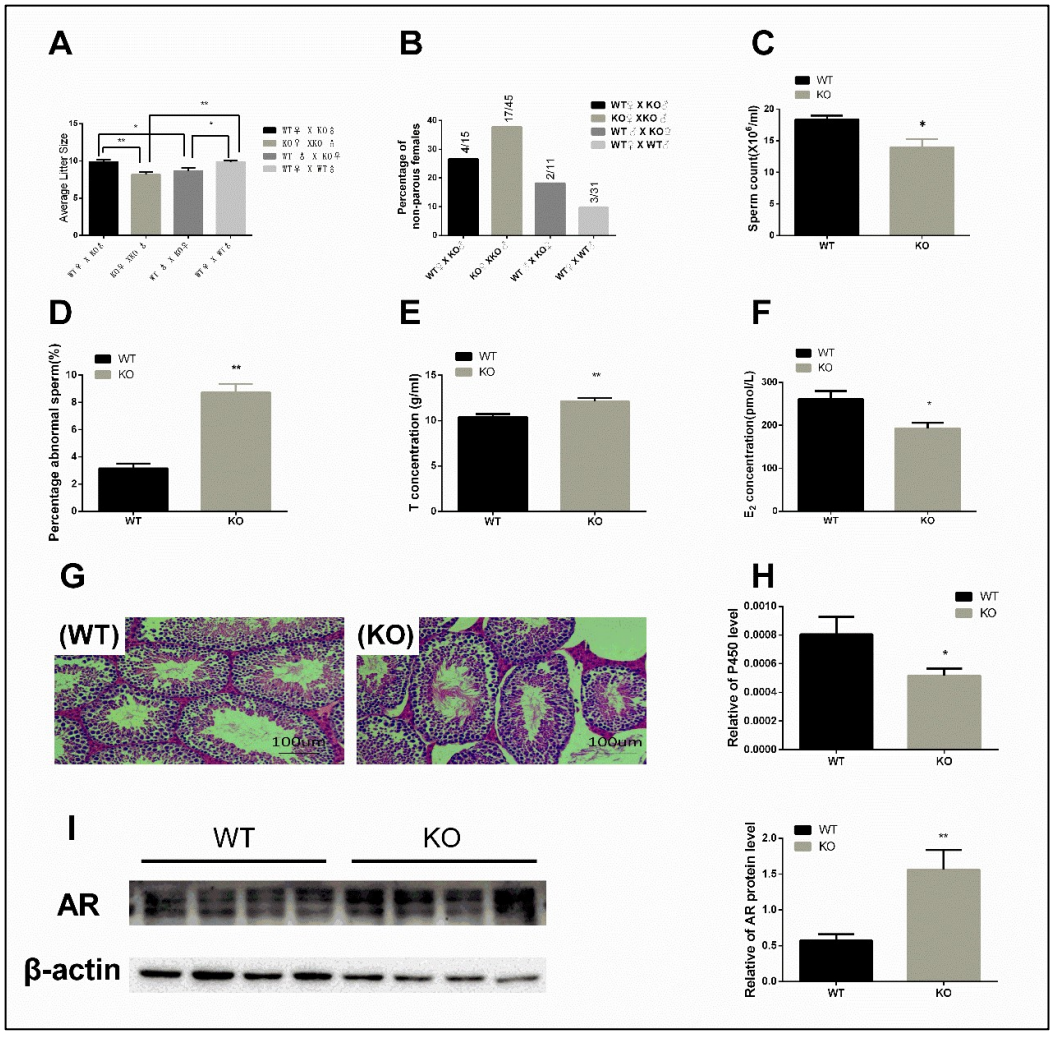

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Figure 2. miR-125b-2 knockout causes different reproduction phenotypes. (A and B) Fertility of male mice. WT and KO males were examined in the following four combinations: WT♀ X KO♂、KO♀ X KO♂、WT ♂ X KO♀、WT♀ X WT♂. The pups (A) and litter rate (B) were counted (n=15-20). (C and D) Effect of miR125b-2 on sperm count (C) and percentage of abnormal sperms (D) (n = 8). T (testosterone) concentration (E) and E2 (estradiol) concentration (F) in 9-week-old WT and KO mice (n=8). (G) Microscopic observations of seminiferous tubules in WT and KO mouse testes: a, Wild-type FVB/NJ male with normal spermatogenesis. b, KO FVB/NJ male (recipient strain). (H) P450 expression in mouse testis in KO mice (n=8). (I) Characterization of AR protein and gray-scale scanning analyses in WT and KO mouse testis (n = 4). All data were expressed as mean ± SEM. *p<0.05, **p<0.01.

2.3. RNA-seq Analysis Revealed Changes in the Mouse Transcriptome in Response to miR125b-2KO

RNA samples from the testis of WT and KO mice were subjected to RNA-seq using an Illumina HiSeq™ 4000 instrument. A total of two samples were analyzed, which included the mixing of three samples for each condition. Totals of 48969660 to 56391004 raw reads were generated in each library, and the valid data were 48561786 to 55911230 reads, with the valid ratios being more than 99% for 2 libraries (the detail characteristics of transcriptome sequencing showed in Supplement File Table S1). The quality evaluation indexes of several sequencing reads suggested that the results were reliable and reasonable, and can meet the requirements of subsequent DEGs screening and data analysis.

We focused on genes differentially expressed between WT and KO mice, including PAP, Yod1, and TSSK1. Among the 2 libraries, 32667 genes were detected. Between the WT_G and KO_G libraries, 324

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significantly differentially expressed genes (p<0.05 and |log2ratio |⩾1) composed of 173 up-regulated and 151 down-regulated genes were identified (Supplemental File Figure S1).

Twenty genes with the most significant differences in up-regulation and down-regulation in KO mouse testis were recorded (Supplemental File Table S2 and S3). To determine the function of DEGs, we mapped them according to terms of the GO database. A total of 1773 genes were categorized into the 3 main categories of GO classification, including biological processes, cellular components, and molecular functions (Supplemental File Figure S2A-C). Among them, the most important biological processes included mitochondrial metabolism, sperm chromatin condensation and other cellular biological processes. To confirm the accuracy of RNA-seq results, expressions of 9 DEGs and one isoform were analyzed by qRT-PCR (Supplemental File Figure S2D). It suggested that the result of RNA-seq were reliable.

2.4. Sperm mtDNA Copy and Integrity

The DEGs of WT and KO involve mitochondrial metabolism biological processes, and others reported that the increase in mitochondria DNA (mtDNA) copy number could result in sperm quality decline. To determine the effect of miR-125b-2 on the mtDNA copy number in mouse sperm, we compared WT with KO mice. We determined the relative transcript level of four marker genes including ATPase6, Cox2,

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Mit-1000 and mt-Cytb. The result showed that all of them increased extremely significantly (p<0.01) in KO group (Figure 3A). The mtDNA copy number depends on the expression level of mt-TFA[17]. So we detected the mRNA level of mt-TFA. As expected, the mRNA level of mt-TFA increased significantly (p<0.01) (Figure 3B). For the changes in the expression of mitochondrial NADH dehydrogenase, we examined the mRNA level of ND1 and ND4 in the sperm, the result showed that in KO mice, the ND1 and ND4 increased significantly in sperm (p<0.001). Consistent with the mtDNA copy number, the mRNA of mt-TFA and ND1 and ND4 level increased significantly in KO group (Figure 3C). To investigate whether the mtDNA integrity and nucleus DNA (nDNA) have changed, we used primers to amplifie MTCYB and MTATP6 sequence to detecte nDNA integrityand mtDNA-1 and mtDNA-2 sequence to detected mtDNA integrity[18]. However, there was no change in nDNA integrity and mtDNA integrity between the KO group and the WT group (Figure 3D-E).

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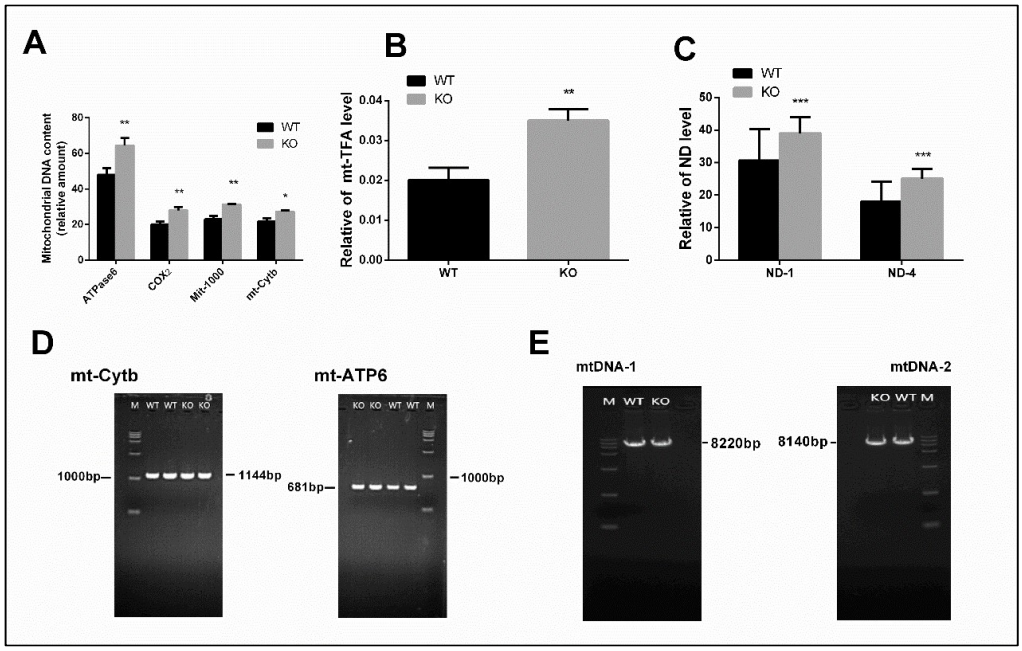

Figure 3. miR-125b-2 affected mtDNA's copy number and integrity. MtDNA copy number (A) mtDNA copy number in the sperms of KO and WT mice. (B) Mitochondrial transcription factor A (mt-TFA) (n=8). (C) Quantifications of ND1 and ND4 mRNA levels in sperm. (D) PCR amplification products of mtCytb (left) and mtATP6 (right) in the sperm of KO and WT mice. (E) Amplification products 8220bp (left) and 8140bp (right) of mtDNA in the sperm of KO and WT mice using long PCR. The asterisk indicates a statistically significant difference at *p<0.05, ** p< 0.01, ***p<0.001.

2.5. miR-125b targeted PAP gene to Suppress Testosterone (T) Secretion in TM3 Cells

2.5.1 miR-125b-5p targets on PAP

Flanking sequence analysis showed that the 3′-UTR of PAP mRNA contains a binding site that perfectly matched the seed region of miR-125b-5p (Figure 4A). To verify PAP is a target of miR-125b-5p. CHO cells were co-transfected with miR-125b-5p mimics/NC and pmirGLO-PAP, pmirGLO-PAP-Mut and pmirGLO-PAP-Del. Forty-eight hours after transfection, the luciferase activity of the WT group were

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assayed, and the pmirGLO group showed the lowest luciferase activity when compared with the other groups (p<0.01). The reduction was rescued in the mutation group and deletion group (Figure 4A). Luciferase activity result showed that PAP was preliminarily the target gene of miR-125b-5p. Therefore, we speculated that miR-125b-5p targeting PAP would impact the spermatogenesis and arrest the formation of sperms. To determine whether miR-125b-5p can interfere with the PAP expression level, we examined the expression level of PAP in mouse testicular tissue after miR-125b-5p knockout. As expected, qRT-PCR analysis showed that KO exhibited higher testis PAP mRNA expression than WT (Figure 4B), and miR-125b-5p knockout increased significantly PAP protein expression (p<0.05) (Figure 4C). These results further verified that miR-125b-5p targeted the 3′-UTR of PAP mRNA, resulting in suppressed expression of PAP protein expression.

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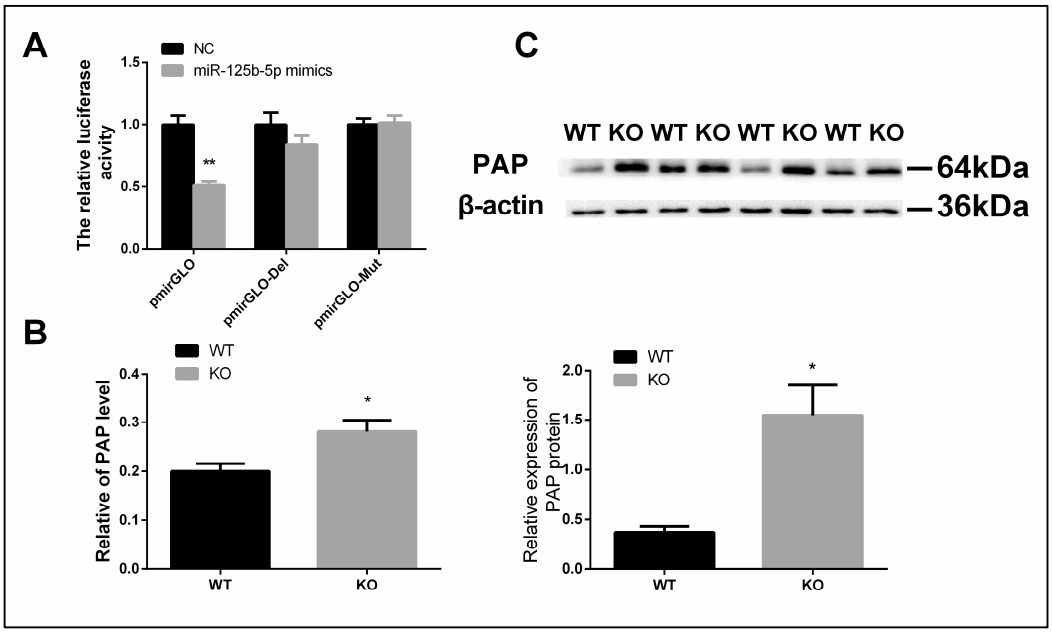

Figure 4. PAP is miR-125b-5p target. (A) PmirGLO dual-luciferase reporter vectors analysis (n=8). Relative luciferase activities were calculated by firefly luminescence/Renilla luminescence. (B) PAP expression in mouse testis after knockout miR-125b-2 (n=8). (C) Characterization of PAP protein and gray-scale scanning analyses in WT and KO mouse testis (n = 4). Values are the mean ± SEM. * $p< 0.05$, ** $p< 0.01$.

2.5.2 miR-125b-2 and PAP Affect the Secretion of Testosterone in TM3

To explore whether PAP is associated with the concentration of testosterone, we used PAP siRNA and miR-125b-5p mimics/inhibitors to transfect TM3 leydig cell line, and then tested the secretion of testosterone. The expression of PAP did not affected by PAP siRNA (Figure 5A). However, PAP1 siRNA and miR-125b-5p mimics can decrease secretion of testosterone significantly while miR-125b-5p inhibitors increased secretion of testosterone markedly (Figure 5B-C). It suggested that there was a relationship between PAP and T secretion.

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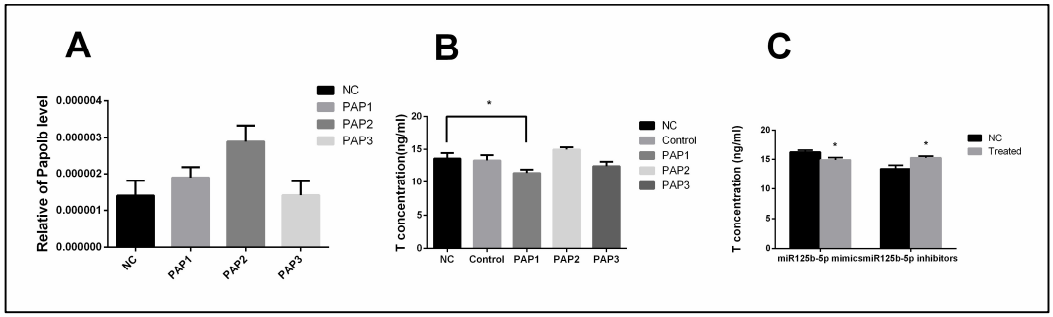

Figure 5. miR-125b-5p and PAP affected TM3 cell secreted testosterone. (A, B) TM3 cell were transfected with different Pap siRNAs and the testosterone levels in the cell supernatant were determined by ELISA. (C) TM3 cells were transfected with miR-125b-5p mimics/NC/inhibitor/iNC. The T of cell supernatant were determined by ELISA (n = 6). All data were expressed as mean ± SEM. *p< 0.05, ** p< 0.01.

2.5.3 The Localization of miR-125b-2 and PAP in Testicular Cells

To verify the direct effects of miR-125b-2 on testicular cells, we isolated different types of testicular cells from WT and KO mice. After purification of isolated cells, we examined the expressions of PAP and miR-125b. Real-time RT-PCR results showed that the miR-125b-2 deleted remarkably decreased the miR-125b levels in GC (p<0.001), but showed no significant differences in LC (p=0.093) and SC (p=0.49) (Figure 6A-C). Interestingly, the PAP were mainly expressed in GC, rarely expressed in LC, and not expressed at all in SC (Figure 6F). Meanwhile, the results showed that the mRNA level of PAP remarkably increased in GC (p<0.001) (Figure 6D), but there was no significant difference in LC (p=0.108) (Figure 6E).

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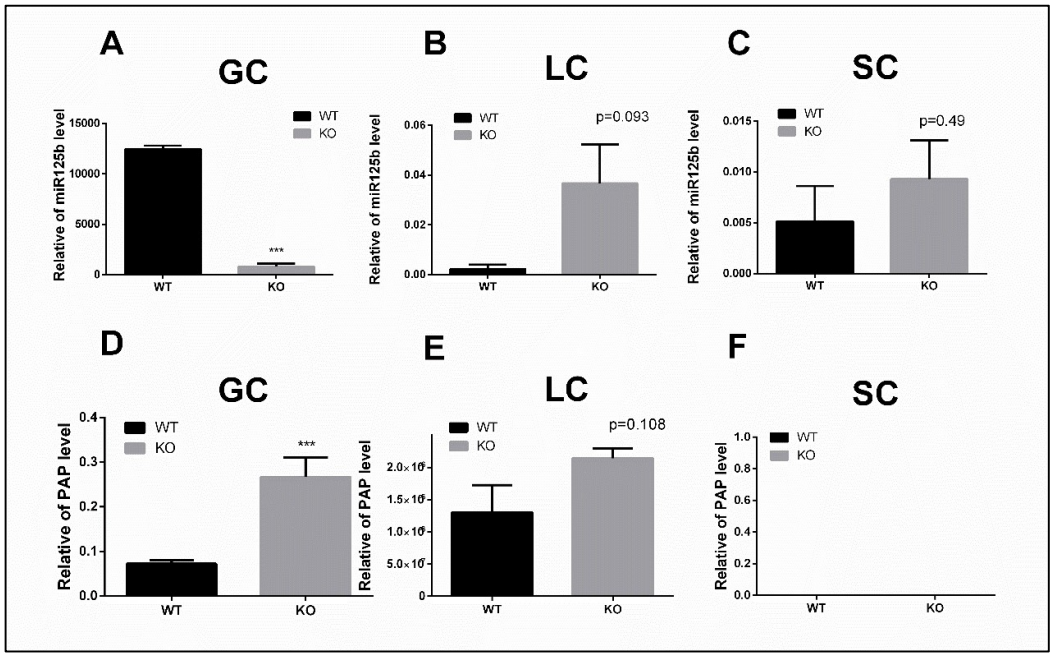

Figure 6. The Relative Expression of miR-125b-2 and PAP mRNA in LC, SC and GC. (A) The relative level of miR-125b in GC. (B) The relative level of miR-125b in LC. (C) The relative level of miR-125b in SC. (D) The relative of mRNA level PAP in GC. (E) The relative mRNA level of PAP in LC. (F) The relative mRNA level of PAP in SC (n=5). Values are the mean ± SEM. *p< 0.05; ** p< 0.01; *** p< 0.001.

3. Discussion

Spermatogenesis is an intricate process of germ cell development and many genes are involved. Any defect in gene expressions or regulations will disrupt spermatogenesis and cause male infertility[19]. Few studies have been conducted on specific microRNA functions in spermatogenesis and male fertility [20, 21]. In the course of breeding KO mice, we found that the number of litter size was significantly reduced and the infertility rate was significantly increased. Similar results have been reported that the miR-125 family plays an important role in regulating maternal genes in oocytes and embryos[15]. Through statistical analysis of litter size and

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litter rate in four mating combinations, we suggested that miR-125b-2 knockout could affect the female and male mouse reproduction performances. In this study, it was validated that miR-125b-2 had a greater influence on litter size of the female mice, and increased infertility of the male mice. We study the effect of miR-125b-2 on male mice, and experiments in female mice are being doing by colleagues. Testis development is tightly regulated process and vital organs for animal reproduction. In this study, RNA-seq and techniques were used to identify the transcriptome differences of testis between the WT and KO mice. The transcriptome analysis identified 324 DEGs between WT and KO groups, 173 up-regulated and 151 down-regulated. Among them, the most important biological processes included sperm chromatin condensation, mitochondrial metabolism and other cellular biological processes. This suggested that miR-125b-2 would affect spermatogenesis and sperm mitochondria.

Sperm quality is highly related to male infertility[22]. Our present study revealed that the sperm count was significantly reduced，the percentage of abnormal sperms was significantly increased and the sperm mitochondria DNA copy number was significantly increased in the KO group compared to the WT group. Mitochondrion is a major organelle that plays an important role in sperm function in physiological conditions[23]. With every unit increase in mtDNA copy number of

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CYTB, there is a 4% decrease in the odds of sperm movement[24]. Others have also reported an increase in mtDNA copy number results in decreased sperm motility. Although the mechanisms regulating mtDNA levels are not fully understood, it has already been proved that changes in TFAM protein levels directly influence mtDNA copy number. Heterozygous knockout mice showed a 50% reduction in mtDNA copy number[25], whereas mice overexpressing the human TFAM protein show a 50% increase in mtDNA levels. Therefore, investigating the sperm mitochondrial genome may provide useful markers of mitochondrial oxidative stress and male fertility. Interestingly, we found the TFAM mRNA level was increased significantly, and it is consistent with the increase of mtDNA copy number in KO mice. And NADH dehydrogenase I (ND1) is used to determine mtDNA copy number which has been reported to be a stable gene in a region of the genome that is rarely deleted[26]. NADH dehydrogenase 4 (ND4) is also commonly used but is more susceptible to deletions due to their location[27]. In our study, we found ND1 mRNA and ND4 mRNA of sperm were increased significantly, which was also consistent with the increase of the mtDNA copy number in KO mice. These three results further illustrated that miR-125b knockout reduced the sperm quality of male mice.

As the result of mRNA-seq, the PAP mRNA level was increased significantly. It has been reported that the PAP, a cytoplasmic poly (A)

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polymerase, is responsible for the additional poly (A) tail extension of specific mRNAs in round spermatids[28-30] and encodes the transcriptional activator CREM, which is highly expressed in male germ cells[31]. To further confirm the function of PAP in testis, we cultured GC, SC, and LC. By RT-qPCR of primary cells of the testis, we found that PAP was mainly expressed in the germ cells, which was consistent with others' reports. Others reported that there was a direct link between the deficiency of a cytoplasmic poly (A) polymerase, PAP, and the arrest of mouse spermiogenesis[28]. Normal fertility and spermatogenesis allows of 2 to 2.5 fold PAP overexpression in mouse[29]. It has been reported that the overexpression of PAP interferes with cell growth and development[32, 33]. Interestingly, our results also support this phenomenon that the overexpression of PAP can reduce the reproductive performance of male mice. PAP was 4.2 fold overexpression in KO compared with WT while the fertility of KO male mice was decreased. The overexpression of PAP in Drosophila results in a dramatic elongation of mRNA poly (A) tails nonspecifically in the cell cytoplasm, which leads to embryonic lethality[33]. These results are consistent with ours in that the overexpression of PAP could lower the reproductive performance. The effect of PAP overexpression on reproduction has not been widely recognized. It can be seen that there is a direct relationship between PAP and reproduction, and the regulatory mechanism of PAP overexpression is

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worth studying.

Testosterone (T) is produced by the LC in response to LH and appropriate secretion of FSH, and LH and T are proved to be fundamentally important for normal spermatogenesis[34, 35]. There was a report that the lowering of testosterone concentrations results in a decrease in overall sexual activity, thoughts, and hot flushes and fantasies. Male patients with androgen deficiency syndromes can take testosterone therapy and it worked pretty well[36]. It is suggested that androgen resistance (high T, elevated LH), or spermatogenesis failure (normal T and LH, elevated FSH), (low testosterone [T] and low or inappropriately normal LH and FSH) [37-39] can define whether the male infertility is due to gonadotropin deficiency and this will have a bad effect on reproduction. In this study, the KO group showed that the T concentration significantly increased while the E_2 concentration significantly decreased. These results demonstrated that miR-125b-2 knockout mice may be in an androgen tolerant environment and lead to the percent of infertility increase in male mice.

All stages of spermatogenesis are dependent on an intimate interaction between Sertoli cells (SC) and Leydig cells (LC), which provide the microenvironment essential for spermatogenesis[5]. Hormone screening can help define whether the male infertility is due to gonadotropin deficiency. Thus, we transfected miR-125b-5p mimics/

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inhibitor/PAP siRNA into LC to detected the difference of T concentration. Interestingly, when TM3 cells transfected with miR-125b-5p mimics/PAP siRNA, the T concentration decreased, and while transfected with miR-125b-5p inhibitors, the T concentration increased. As the results of Western blotting and Luciferase Assay mentioned above, miR-125b-5p decreased T concentration of male mice testis by targeting PAP.

In conclusion, we demonstrated miR-125b-2 could regulate T secretion by targeting PAP and increased sperm mtDNA copy number, thereby decreasing reproductive performance in male mice. Our findings provide a better understanding of the molecular mechanism of infertility, and the possible regulatory role of miR-125b-2 in spermatogenesis and fertilization, which could be applied to clinical andrology and breeding management.

4. MATERIALS AND METHODS

4.1. Ethics Statement

The animal experiments complied with the guidelines of Guangdong Province on the Review of Welfare and Ethics of Laboratory Animals approved by the Guangdong Province Administration Office of Laboratory Animals. All procedures were conducted according to the protocol (SCAU-AEC-2010-0416) approved by the Animal Ethics

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Committee of South China Agricultural University.

4.2. Fertility Test

MiR-125b-2 gene knockout model of mice was constructed by CRISPR/Cas9 System (Cyagen Biosciences Inc, USA). Fertility was assessed by mating experimental males. The mice were allowed to mate for a 30-day period, and pairs were monitored regularly for signs of pregnancy. The pregnancy ratio was calculated by the number of pregnant female mice undergoing parturition over the total female mice in each group.

4.3. Body Weight Measurement

Two groups (WT and KO) of 18-18.7g male FVB/N mouse were studied (8 animals/group). The mice were housed individually for 1 week to get acclimatized to the new environment. Body weight was measured for every week following acclimatization for 2 months.

4.4. Morphological Observation of Testis

Four mice of every group were selected randomly for testicular histopathology. The left testis was fixed in 4% paraformaldehyde overnight, and then washed with 70% ethanol and Li_2CO_3-saturated solution. Following standard procedures for paraffin-embedded tissues, testis tissues were sliced into 5μm sections and stained by hematoxylin-eosin staining (HE) according to a standard protocol for

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histopathologic analysis[40].

4.5. Sperm Quality Evaluation

The cauda epididymis was isolated after the mice were sacrificed, and transferred into small beaker containing 1ml pre-warmed 37 °C normal saline and excised with fine scissors. After 10 min of incubation, spermatozoa were allowed to swim out freely, and undissolved tissues were removed. Aliquots of sperm suspensions were prepared for the determination of sperm parameters, including count and morphology, which were evaluated as previously described[41]. Briefly, a 10-mL sperm suspension was analyzed to determine the spermatozoa count using a hemocytometer. A 10mL aliquot of sperm suspension was used to assess sperm morphology. The suspension was uniformly smeared onto a glass slide, air-dried, and fixed with methanol for 5 min. Spermatozoa were then stained with eosin for 1 h and washed with distilled water. Slides were air-dried at room temperature, and sperm morphology was examined using a light microscope to study 2000 sperm cells under 400X magnification.

4.6. Target Prediction and Pathway Analysis

We predicted target genes of miRNA in mouse at the genome level. In brief, the 3'-UTR sequences of murine transcripts in the whole genome were obtained from Ensemble genome browser 80 (sscorfa10.2;

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http://www.ensembl.org/Sus_scrofa/). Mature differentially expressed miRNAs sequences were downloaded from miRbase release 21 (http://www.mirbase.org), and RNA hybrid software (http://www.bibiserv.techfak.uni-bielefeld.de/rnahybrid) was used to analyze miRNA targets by using its own algorithm. Our prediction was restricted to a perfect match of the seed region (2 – 7 bases of the miRNA 5' end; G:U matches were permitted), due to the importance of the seed sequence for miRNA-mRNA binding[42]. In addition, the matches were restricted to those with less than 220 kcal/mol of low free energy in the binding of miRNA-mRNA. Furthermore, the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 online service (http://david.abcc.ncifcrf.gov/)[43] was used for Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis based on all differentially expressed miRNA potential targets, and the minimum number of genes of potential pathways affected by differentially expressed miRNAs was 20. Individual GO analysis and KEGG analysis of miR-125b-5p were also conducted based on the potential targets of miR-125b-5p.

4.7. Protein Extraction and Western blot

Testicular tissue was lysed with RIPA buffer with protease inhibitors. Total soluble protein was quantified by the BCA protein assay. Total

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protein (30ug) was loaded onto a 10% SDS-PAGE gel, separated by electrophoresis, and transferred onto a polyvinylidene difluoride membrane. Blots were blocked with 5% skim milk and incubated with primary antibody overnight at 4°C, followed by incubation with secondary antibody for 1 h at room temperature, and measured with an Infrared Imaging System (LI-COR, Lincoln, NE). Protein expression was normalized by detection of β-actin (Abcam, Cambridge, UK). Protein from testicular tissue feed with ordinary daily food to 10 weeks was isolated to measure the expression level of PAP-β, and probed using the primary monoclonal antibody (Santa Cruz, California, UK).

4.8. Plasmid Construction, Transfection, and Luciferase Assay

To generate the mouse PAP 3'-UTR sequence containing miR-125b target site CTCAGGG, 2 single-stranded DNA were synthesized (Sangon, Shanghai, China) and annealed to form a double-stranded DNA, which contained the recognition sites of restriction enzymes XbaI and XhoI. The synthetic sequence was inserted into XbaI and XhoI enzyme-digested vector pGL3-Control (Promega Co., Madison, WI), downstream of the luciferase gene. Meanwhile, the deleted and mutagenic PAP 3'-UTR reporter vectors were constructed with 5 exchanged nucleotides or a deleted target site in the same way. The sequences of 3 types of 3'-UTR were as follows: PAP 3'-UTR (sense,

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tcgagTCTACTCTTTTACCAAGTCTCAGGGATACTATAAATTAGAGC Tt; antisense, ctagaAGCTCTAATTTATAGTATCCCTGAGACTTGGTAAAAGAGTA GAc), PAP 3'-UTR mut (sense, tcgagTCTACTCTTTTACCAAGTGAGACGTATACTATAAATTAGAGC Tt; antisense, ctagaAGCTCTAATTTATAGTATACGTCTCACTTGGTAAAAGAGTA GAc), and Pap 3'-UTR del (sense, tcgagTCTACTCTTTTACCAAGTATACTATAAATTAGAGCTt; antisense, ctagaAGCTCTAATTTATAGTATACTTGGTAAAAGAGTAGAc).

Chinese hamster ovary (CHO) cells were seeded at a density of 4×10^4 cells per well in 96-well plates. When the cells reached 60 to 70% confluence, reporter vector pmirGLO-PAP, pmirGLO-PAP-Mut and pmirGLO-PAP-Del were co-transfected with the miR-125b-5p mimic or negative control (NC). GenEscort II (Huiji, Nanjing, China) were used to mediate the transfection procedure according to the manufacturer's protocol. Transfection efficiency was normalized by the activity of renilla luciferase expressed from a co-transfected pRL-TK vector. The luciferase assay was performed with the Dual-Luciferase reporter assay system (Promega Co.).

4.9. RNA Extraction and qRT-PCR

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Total RNA was extracted from testicular tissues using Trizol reagent. RNA concentration was determined using the NanoDrop 2000. Total RNA (2ug) was reverse-transcribed to cDNA using Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI) with OligodT18 (IR) or Universal Adaptor Primer (miRNAs) from the One Step PrimeScript® miRNA cDNA Synthesis Kit. After 1 h of incubation at 42°C and 10 min of deactivation at 75°C, the reaction mixes were used as the templates for PCR. Real-time PCR was performed with standard protocols on the STRATA-GENE Mx3005P sequence detection system. The PCR mixture contained 2ul of cDNA, 10ul of 2× SYBR Green PCR Master Mix, 0.5ul of each primer, and water to make up the final volume to 20ul. The reaction was performed in a 96-well optical plate at 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, optimal reannealing temperature for 15 s and 72°C for 40 s. All reactions were run in duplicate, and a negative control (NC) without template was included for each gene. The primers of PAP were showed as follows: the upstream and downstream primers of PAP were 5'-TGATCGAGACCCTCCAGC-3' and 5'-CTCAATTACAGCTTGTGGAAGATTC-3'. Primers were designed based on the sequence of each gene by using Premier 5.0.

4.10. Sperm mtDNA Integrity

The mitochondria of sperms were extracted by QIAamp® DNA Mini

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Kit. The mtDNA integrity was carried out following a previous study with minor modifications[44]. Briefly, long PCR amplification was applied to 8220 and 8140bp out of the 16kbp mitochondrial genome. Primers of mtDNA fragment were shown in Table 2, designed and synthesized by Sangon (Sangon Co., LTD Shanghai, China). Amplification conditions were as follows: 30 cycles of denaturing (98℃ for 30s), annealing (55℃ for 15 s), and extension (68℃ for 9 min). PCR products were examined by electrophoresis with 1% agarose gel and visualized by 4S Red Plus Nucleic Acid Stain (Sangon Co., LTD Shanghai, China).

Gene	Primer sequences	Accession No.	Product sizes (bp)
mtDNA-1	F:GTTAATGTAGCTTAATAACAAAGCAAAGC R:TAGTTGGGTAGTAGGTGTAAATGTATGTG	NC_005089.1	8220
mtDNA-2	F:ATTGGATCAACAAATCTCCTAGG R:TTGTTAATGTTTATTGCGTAATAGAGTATG	NC_005089.1	8140

Table 2. Primer sequences and their corresponding PCR product sizes for long PCR.

4.11. Sperm mtDNA Mutations of mt-Cytb and mt-ATP6

Mutations of mt-Cytb and mt-ATP6 were evaluated by PCR and sequence analysis by Mao et al[45]. Table 3 shows the primer sequences, which were designed and synthesized by Sangon (Sangon Co., LTD Shanghai, China). PCR amplification conditions were as follows: 94℃ for 5 min, followed by 30 cycles at 94 ℃ for 30 s, 54 ℃ for 15 s (MTCYB, MTATP6), and 72 ℃ for 80 s (MTCYB, MTATP6). Mutations in PCR-amplified products were analyzed by blast analysis.

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Gene	Primer sequences	Gene ID	Product sizes (bp)
Mt-Cytb	F:ATGACAAACATACGAAAAACACA R:ATGGATATAATTTTAGTATTTTGTCTTCGA	R17711	1144
Mt-ATP6	F: ATGAACGAAAATCTATTTGCCTC R:TTATGTATTATCATGTAGATATAGGCTTACTAGGA	17705	681

Table 3. Primer sequences and their corresponding PCR product sizes for mutations of mt-Cytb and mt-ATP6.

4.12. siRNA transfection

Three siRNAs oligos target to PAP gene and negative control were ordered from Guangzhou RiboBio Co (Supplemental File Table S4). siRNA oligos were transfected into mouse TM3 cell using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions and the most effective siRNA was chosen for subsequent analysis. After transfection with the most effective siRNA or NC, the levels of testosterone in supernatant were measured.

4.13. Cell Isolation and Purification

Leydig cells (LC) were isolated according to the protocol described previously[46, 47]. Briefly, testes from 3 week-old WT mice were collected and washed by PBS. Then the tunica-free testes were incubated with 0.25 mg/mL collagenase type I (Sigma, US) at 32℃ for 10 min with a gentle swing. The suspensions were allowed to settle naturally and filtered through 75 mm copper meshes to separate interstitial cells and

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seminiferous tubules. The interstitial cells were cultured in F12/DMEM (Sigma, US) with 100 U/mL penicillin, 100 mg/mL streptomycin, 10% FBS (Gibico, US). After 15 min, the non-adherent and loosely adherent cells were collected and resuspended by F12/DMEM with 10% FBS, 100 U/ mL penicillin, 100 mg/mL streptomycin and cultured at 5% CO2 at 34℃.

The seminiferous tubules were re-suspended in PBS, collagenase type I at 32℃ for 15 min to remove testicular peritubular myoid cells (TPC). The tubules were broken into small pieces and incubated with 1 mg/mL hyaluronidase (Sigma, US) at 32℃ for 10 min with gentle pipetting to separate germ cells (GCs) and Sertoli cells (SCs)[48]. Suspensions were cultured with F12/DMEM at 32℃ for 6 hr. The GCs were recovered by collecting non-adherent cells. SCs were cultured for an additional 48 hours and then treated with a hypotonic solution (20 mM Tris, pH 7.4) for 2 min to remove GCs[49].

4.14. Statistical Analysis

All experiments included at least five replicates per group. Data were evaluated by Student's t-test or one-way ANOVA analysis, and differences between groups were considered statistically significant at $p<0.05$. All statistical analyses were performed with PASW Statistics 18 software.

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Supplemental Information

	Raw Data		Valid Data		Valid Ratio%			
Sample	Read	Base	Read	Base	Reads	Q20%	Q30%	GC content%
WT_G	56391004	8.46G	55911230	8.39G	99.15	99.66	94.90	48
KO_G	48969660	7.35G	48561786	7.28G	99.17	99.57	94.03	48

Table S1. Characteristics of the reads from 2 different mouse testis. A total of two samples were analyzed, which included the mixing of three testes for each condition (WT and KO).

Gene name	Description
mt-Nd2	mitochondrial encoded NADH dehydrogenase 2
Actrt2	actin-related protein T2
Kif2b	kinesin family member 2B
Gtsf1l	gametocyte specific factor 1-like
Gm5617	predicted gene 5617
Cetn1	centrin 1
Gm9795	predicted pseudogene 9795
Papolb	poly (A) polymerase beta (testis specific)

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4930407I10Rik	RIKEN cDNA 4930407I10 gene
Hypm	huntingtin interacting protein M

Table S2. Ten genes with the most significant differences in up-regulation in KO mouse testis.

Gene name	Description
Smcp	sperm mitochondria-associated cysteine-rich protein
mt-Cytb	mitochondrial encoded cytochrome b
Hils1	histone H1-like protein in spermatids 1
Gk2	glycerol kinase 2
Eid3	EP300 interacting inhibitor of differentiation 3
Hoga1;4933411K16Rik	4-hydroxy-2-oxoglutarate aldolase 1 Symbol; Acc:MGI:1914015]
Prm3	protamine 3
1700003E24Rik	RIKEN cDNA 1700003E24 gene
4930403D09Rik	RIKEN cDNA 4930403D09 gene

Table S3. Ten genes with the most significant differences in down-regulation in KO mouse testis.

Product name	Target sequence
PAP siRNA1	CCACCTAAGCCTACAATGA
PAP siRNA2	CCCATAGAAAGCTCAGGAA
PAP siRNA3	GAAATACAGCAACGAACAT

Table S4. siRNA oligos target to PAP gene were synthesized.

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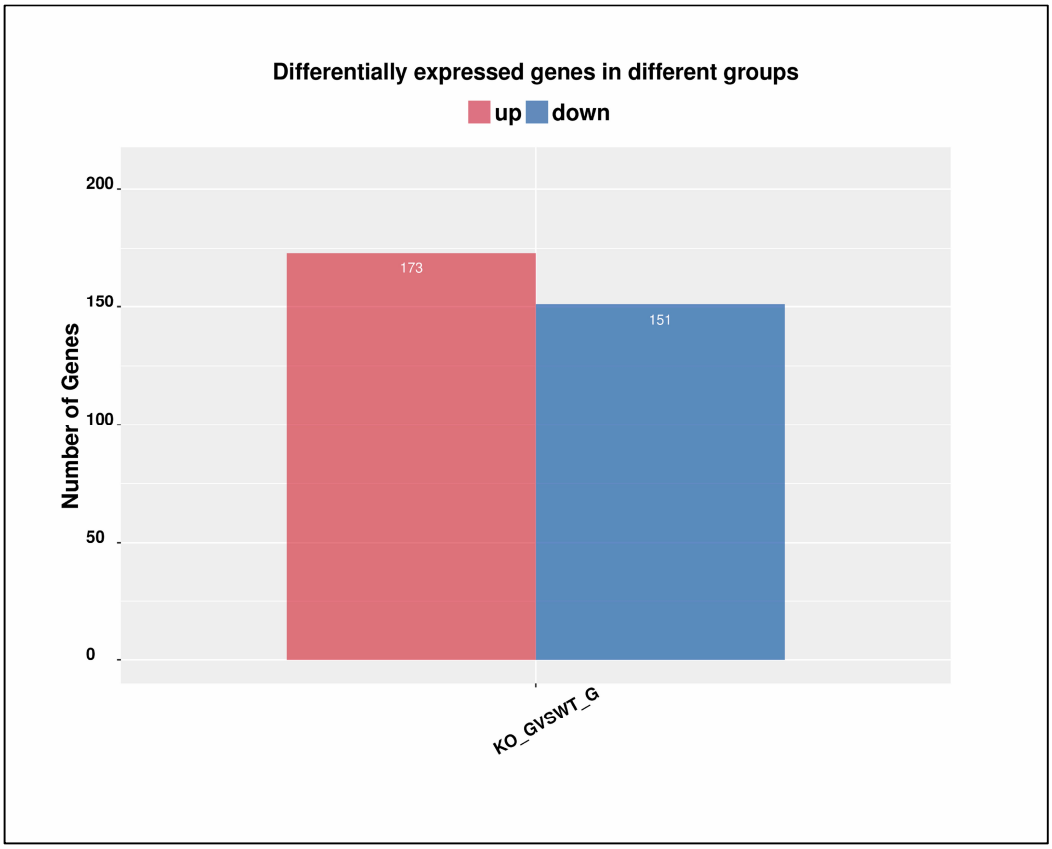

Figure S1. Up-regulated and down-regulated genes number between KO mice and WT mice.

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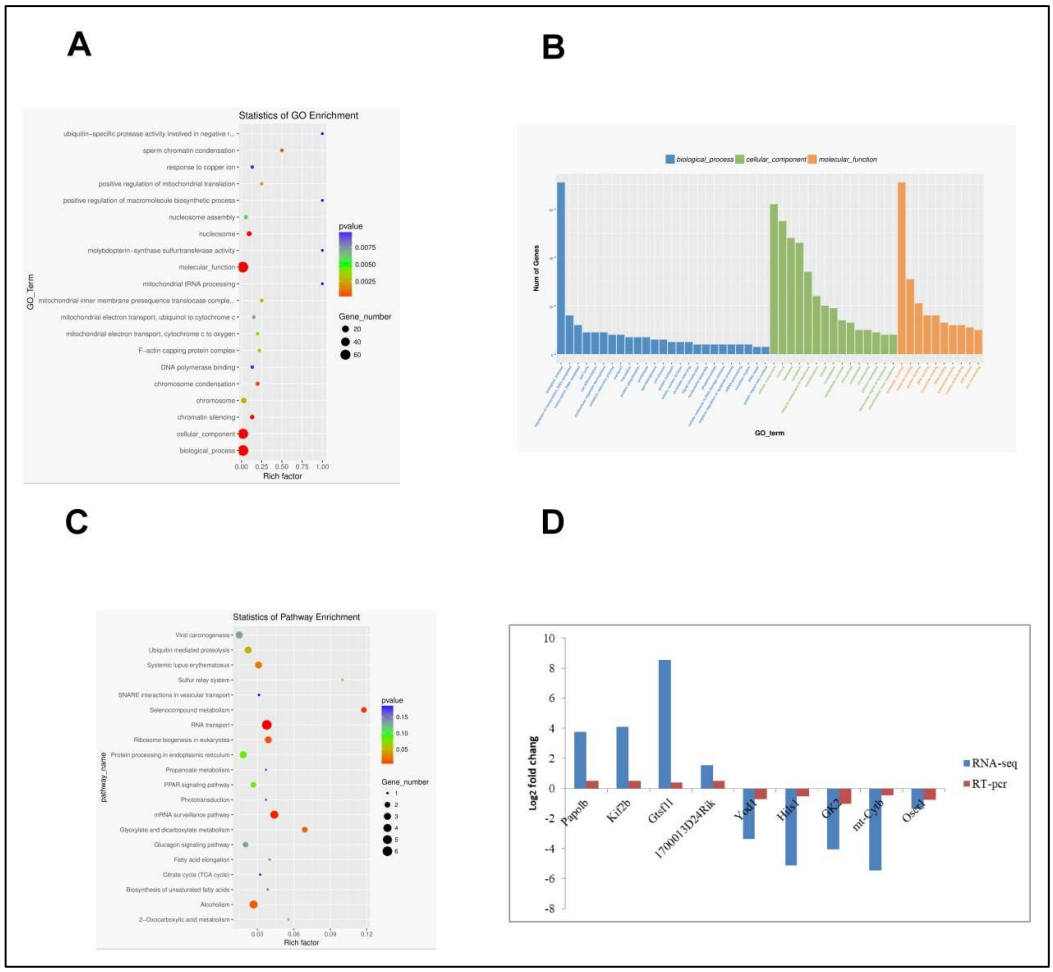

Figure S2. Analysis of differentially expressed genes in WT and KO testes. (A, B) Analysis of biological functions of GO-enriched genes from KO and WT. (C) KEGG pathway enrichment analysis of differentially expressed genes from KO and WT. (D) Validation of relative gene expression obtained from RNA-seq by qRT-PCR (n=6). Relative expression values of qRT-PCR are presented as an average.