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Inactivation of *Dicer1* has a severe cumulative impact on the formation of mature germ cells in mouse testes

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ABSTRACT

Dicer1, an RNase III endonuclease, is indispensable for the maturation of miRNA and siRNA, which control gene expression through the RNAi pathway. The diverse functions of miRNA involving multiple developmental processes have been elucidated, but the role of *Dicer1* in spermatogenesis is just beginning to be revealed. Mice lacking *Dicer1* were reported to be embryonic lethal at E7.5. In the present study, mice with a *Dicer1* conditional allele were crossed with *Vasa-cre* transgenic mice to delete *Dicer1* as early as the prospermatogonia stage (at E15). At P40, seminiferous tubules of *Dicer1* deficient mice showed several aberrant phenotypes. A large number of apoptotic germ cells were detected by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, but several events in meiosis of spermatocytes appeared unaffected. The mutant mice were found to be sterile, likely due to the extensive decrease in number and morphological abnormalities of mature sperm in the epididymis, which, together with the numerous haploid cells in the testis, indicated a severely affected transition from round to functional elongated spermatozoa. Additionally, we found milder phenotypes when *Dicer1* was inactivated in later stages of spermatogenesis in *Stra8-cre* and *Pgk2-cre* transgenic mice. In conclusion, our findings suggest that the loss of *Dicer1* has a continuous and cumulative effect on the process of spermatogenesis and blocks the germ cells in the stage of round spermatids to a large extent, ultimately leading to the generation of abnormal sperm.

1. Introduction

Spermatogenesis is an orderly and continuous process that is highly regulated by multiple genes. It has been previously demonstrated that the process of spermatogenesis is subjected to post-trancriptional regulation, in which miRNAs are predicted to play important roles [1,2]. MiRNAs are small non-protein-coding RNAs which negatively regulate the gene expression profile at a post-transcription level by repressing gene translation or degradation of target mRNAs [3–7]. Since it has been predicted that >60% of human protein-coding mRNAs cannot escape the selective pressure by pairing with miRNAs, almost every biological function and process is influenced, to some extent, by miRNAs [8,9]. In fact, previous studies have revealed critical functions of miRNAs in such processes as stem cell proliferation and self-renewal, limb formation, regulation of lung epithelium morphogenesis, hair follicle development, embryonic angiogenesis, T-cell differentiation and female fertility [10–16]. The fact that several miRNAs have been shown to be specifically expressed in the male germ-line may implicate their critical role in different steps of spermatogenesis or male fertility [17–19].

Dicer1, an RNase III endonuclease, is responsible for processing pre-miRNAs into miRNA duplexes in the cytoplasm and has been reported to play a pivotal role in miRNA maturation [20]. *Dicer1*-null mutant mice show complete loss of functional miRNAs and fail in embryonic development in E7.5 [21]. *Dicer1* depletion in oocytes leads to arrested oogenesis in meiosis I. The function of *Dicer* in the process of spermatogenesis, however, is just beginning to be unraveled using a *Dicer1* conditional knockout allele. Two groups (Hayashi and Maatouk) have independently carried out experiments using *TNAP-cre* transgenic mice to mutate *Dicer1* function as early as the PGC stage [17,22]. The mutant mice were found initially subfertile and infertile when older than 8 months due to their spermatogenesis being mostly arrested at the stage of round spermatids. The fact that *TNAP-cre* expression efficiency was only about 60% in germ cells and exhibited severe ectopic expression complicated the interpretation of the experimental results [23]. Therefore, we employed in our study transgenic mice which possessed a much more specific and efficient expression pattern of *Cre*-recombinase from the *Vasa* locus to delete *Dicer1* function at the stage of prospermatogonia to confirm and further explore the role of *Dicer1* in spermatogenesis. To assess the impact of *Dicer1*

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depletion on postnatal spermatogenesis, we also inactivated *Dicer1* at two different stages in spermatogenesis by taking advantage of *Stra8-cre* and *Pgk2-cre* transgenic mice. We found that *Dicer1* was required for spermatogenesis, especially in spermiogenesis, and deletion of *Dicer1* function at different stages led to dissimilar phenotypes.

2. Methods and materials

2.1. Animal strains and crosses

Dicer1flox mice were generated by a previously described method [24]. *Vasa(Ddx4)-cre* was previously characterized [25]. To achieve specific deletion of *Dicer1* genes in germ cells, female mice, which were homozygous for Dicer1flox alleles, were crossed with male *Vasa-cre* mice. The male heterozygous progeny, with the genotype of $Dicer1^{flox/wt}$;*Vasa-cre,* were mated with female Dicer1-flox mice to finally obtain the $Dicer1^{flox/flox}$;*Vasa-cre* mice. The $Dicer1^{flox/wt}$; *Vasa-cre* or $Dicer1^{flox/flox}$ littermates were used as control groups. The $Dicer1^{flox/flox}$;*Stra8-cre* and $Dicer1^{flox/flox}$; *Pgk2-cre* mice were generated using the same method.

2.2. Histology and immunofluorescence

Testes were dissected and fixed in Bouin fixative solution (PFA for terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) and immunofluorescence assays) overnight at room temperature. On the following day, tissues were dehydrated through an ethanol series and then cleared in xylene. Paraffin was used to embed the tissues, and 4 μm sections were prepared for hematoxylin-eosin (H&E) staining and immunofluorescence assays, which were carried out according to the instructions of the manufacturer of primary antibodies. The results were examined using a fluorescence microscope (Olympus). The following primary antibodies were used: anti-*SCP3* (1:50, Abcam), anti-*γH2AX* (1:400, Abcam) and anti-phospho-histone H3 (1:200, CST). The TUNEL assay was performed to detect apoptotic cells with the Promega DeadEnd™ Fluorometric TUNEL System.

2.3. RNA preparation and quantitative real-time PCR

Tissues were stored at −80 °C after dissection until used for RNA extraction using TRIZOL® following the instructions of the manufacturer (Invitrogen Life Technologies). Reverse transcription was then performed using the M-MLV-reverse transcript system (Promega) and random primers provided by Invitrogen to obtain the cDNA product. Real-time PCR was performed on the ABi PRISM® Real-time PCR system, following the methods from TaKaRa. Relative quantities were normalized against *β-actin*. The results were analyzed with SDS 1.4 software from Applied Biosystems. The primers were listed in Table S1.

2.4. Sperm count and analysis of morphology

The caudal epididymides from adult mice were carefully removed, washed with PBS, cut with scissors, squeezed with

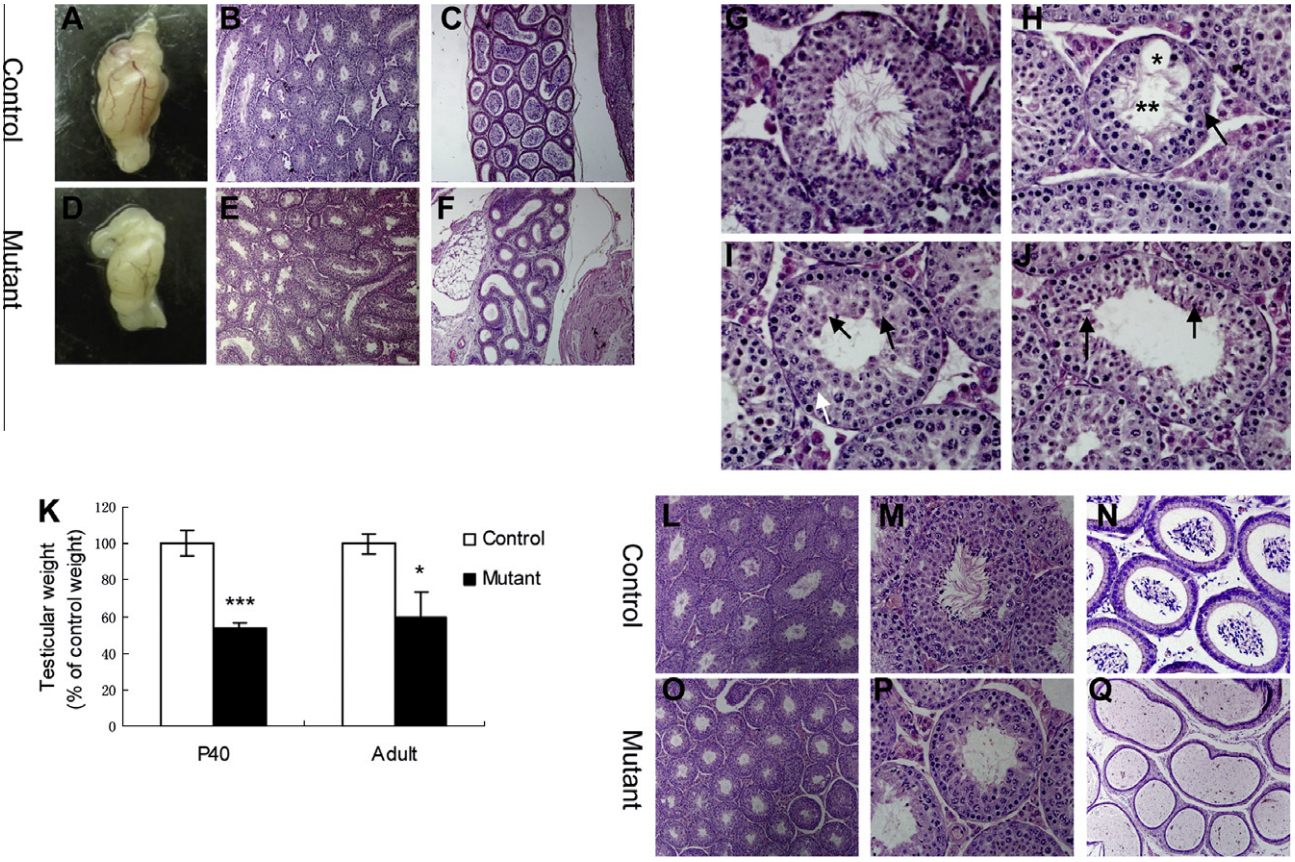

Fig. 1. Reduction in testis size and weight, abnormalities in the seminiferous tubules of *Vasa-cre;* $Dicer1^{flox/flox}$ (mutant) testes**.** Testes from mutant mice at P40 showed significant reduction in size (A and D) compared to the controls. Mutant testes nearly completely lacked mature spermatozoa as observed by H&E staining of seminiferous tubules (B and E) and epididymides (C and F). Mutant tubules exhibited vacuolation (∗ in H), incorrect placement of cell types (arrow in H) and lack of elongated spermatozoa (∗∗ in H). A number of round spermatids (arrows in I) and mis-shapened elongated spermatozoa heads (arrows in J) could be observed in mutant tubules. (K) Weight of mutant testes largely decreased. (L–Q) Testes from mutant mice at P120 did not show a more severe phenotype compared to mutant mice at P40, but a large number of exfoliated cells appeared in the epididymis of mutant mice (Q). Results are mean ± SEM (n = 3 animals/genotype), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus controls.

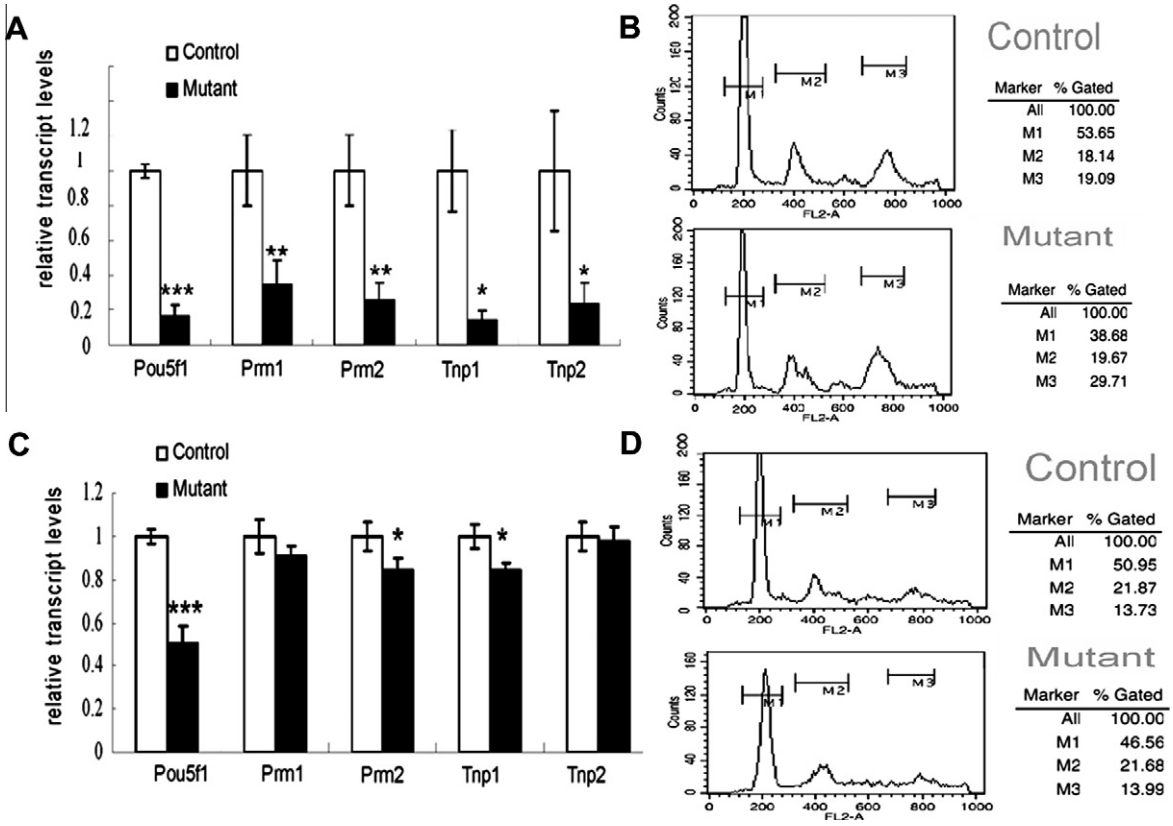

Fig. 2. *Dicer1* deficient mice exhibit decreased capability in generating haploid germ cells. Quantitative real-time PCR detection of several genes expressed in spermatogonia and haploid germ cells of P40 testes (A) and P210 testes (C) illustrate insufficient generation of spermatogonia and haploid cells in the first round of spermatogesis. Flow cytometric analysis of haploid, diploid and tetraploid cells in P40 (B) and P210 (D) testes. M1, M2 and M3 populations correspond to 1C, 2C and 4C cells respectively. Results are mean ± SEM ($n = 3$ animals/genotype), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus controls.

forceps in 200 μl MHTF (Modified Human Tubal Fluid) and incubated in 37 °C for 5 min in order to release the sperm cells thoroughly. The total sperm cells were estimated by a hemocytometer.

For the sperm morphology analysis, sperm suspended in MHTF were spread onto glass slides, dried in room air, fixed in 4% PFA for 60 min, washed with PBS for 5 min and then subjected to H&E staining. More than 200 cells were examined for the morphological analysis.

3. Results

3.1. Mice with Dicer1 deleted in testes show dysfunctional spermatogenesis

The method for producing *Dicer1* mutant mice is described above. The *Dicer1* transcript level from the mutant testes at P40 (just after the first wave of spermatogenesis was completed) was largely decreased, confirming the high knockout efficiency (Supplementary Fig. S1). *Dicer1* deficient mice were distinguished from control littermates by the testes, which were decreased by nearly half in size and weight (Fig. 1A, D and K). Furthermore, H&E staining of the sections revealed that, compared to the control groups, the P40 mutant mice lacked elongated spermatozoa in the majority of seminiferous tubules and epididymides. Some tubules showed severe disorganization, vacuolation and incorrect localization of round spermatids. Additionally, the pachytene spermatocytes and many a round spermatids could still be observed in the tubules of mutant mice (Fig. 1B–J). Testes of adult mutant mice were also histologically analyzed and showed no progressively aberrant development compared to mutant testes at P40, except that a large number of exfoliated cells were found in the epididymis, which indicated the sloughing of testicular cells and some premature sperm cells (Fig. 1L–Q).

Cell death has been detected both in vivo and in vitro due to the loss of *Dicer* [10,24,26]. To determine whether conditional *Dicer1* knockout in the testis leads to cellular apoptosis, testis sections of P40 and adults mice were analyzed by the TUNEL assay. A large number of cells adjacent to the seminiferous tubules basement membrane exhibited a strong signal (Fig. S2). These results may partially explain the significant reduction in testis size of the *Dicer1* mutant mice.

3.2. The emergence of haploid cells was affected by the loss of Dicer1 in testes

Since a number of round spermatids could still be observed in mutant tubules, further investigations were required to determine whether the *Dicer1* mutant mice showed decreased capability in generating haploid cells. We examined the mRNA levels of four haploid markers in testes of mice at P40 by quantitative real-time PCR. Compared to the control group, the haploid-specific transcripts of *Vasa-cre; Dicer1$^{flox/flox}$* mice were largely decreased (Fig. 2A). These results were consistent with those of flow cytometry analysis, which was performed to evaluate the testicular cell populations at P40 (Fig. 2B). Additionally, the downregulated transcript level of the *Pou5f1* (*Oct4*) gene in the mutant testes indicated a disturbance in the maintenance of the spermatogonial stem cell pool.

We also examined the transcript levels of haploid markers in the adult experimental groups (P210), which surprisingly were not as significantly downregulated as in the P40 groups (Fig. 2C). Flow cytometry analysis also demonstrated a slight decrease in the population of haploid cells (Fig. 2D).

Table 1
Vasa-cre; Dicer1$^{flox/flox}$ male mice are infertile.

Male (age/months)	Vaginal plugs	Litters	Pups
Mutant 1 (4)	3	0	0
Control 1 (4)	3	3	22
Mutant 2 (2)	4	0	0
Control 2 (2)	4	4	30
Mutant 3 (7)	3	0	0
Control 3 (7)	3	3	21

3.3. Dicer1 is required for mouse fertility

Since the *Dicer1* germline mutant mice exhibited abnormal production of haploid cells and mature spermatozoa compared to the control groups, we then tested whether the *Dicer1* germline mutant mice possessed a normal ability to sire offspring. Adult mutant mice at different ages and control groups were mated with female wild-type *ICR* mice. Each female ICR mouse was checked daily for the vaginal plug to confirm the occurrence of mating. The results showed that the *Dicer1* deficient mice were sterile, while mice of control groups exhibited normal fertility (Table 1).

3.4. Meiosis of spermatocytes in Dicer1 deficient testes

During normal spermatogenesis, functional haploid cells are generated through two successive phases of meiosis of spermatocytes, in which prophase I is divided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis. Certain biochemical events in prophase I (e.g. synapsis of homologous chromosomes, formation of synaptonemal complexes, homologous recombination) are critical for meiosis and spermatogenesis. Although the histological analysis of *Dicer1* mutant testes sections revealed no discernible abnormalities in meiotic spermatocytes, whether biochemical events in meiosis are affected by the depletion of *Dicer1* remained to be determined. Sections of testes at P120 were immunostained to assess the presence of synaptonemal complexes using the *SCP3* (synaptonemal complex protein 3) antibody and to detect the transition from zygotene to pahcytene by double-staining with the antibody against $\gamma H2X$, which localizes to the whole nucleus in zygotene before it remains only in the XY body in pachytene. However, no distinct alterations were observed compared with the control littermates (Fig. S3A). Additionally, immunostaining of phosphorylated serine 10 of histone H3 (a marker of chromatin condensation in mitosis and meiosis) to detect the presence of meiotic metaphase in the mutant tubules showed no difference compared with controls (Fig. S3B).

3.5. Ablation of Dicer1 causes morphological defects and decreases the number of elongated spermtozoa

Previously, *Dicer1* deficient mice, generated by Maatouk using the *TNAP-cre* transgene with only approximately 60% *cre* activity in the germ cells, showed subfertile and not a total lack of sperm cells in the epididymides [22]. Here, *Dicer1* deficient mice created by employing mice carrying the *Vasa-cre* transgene, which exhibited >95% of *cre* activity in germ cells at birth, were sterile. Whether the complete lack of mature spermatozoa led to the loss of ability to generate progeny needed to be determined. Surprisingly, a number of elongated spermatozoa were released when we carried out the sperm counting assay described above. Additionally, compared to the control group, the number of sperm cells released from the epididymides of *Dicer1* mutant mice were notably decreased by approximately 50-fold (Fig. 3A). Moreover, the percentage of morphologically abnormal spermatozoa, showing globozoospermia, loss of acrosome, and morphological defects of flagellum, significantly increased (Fig. 3B and C). The obvious decrease in number of spermatozoa and large scale morphological

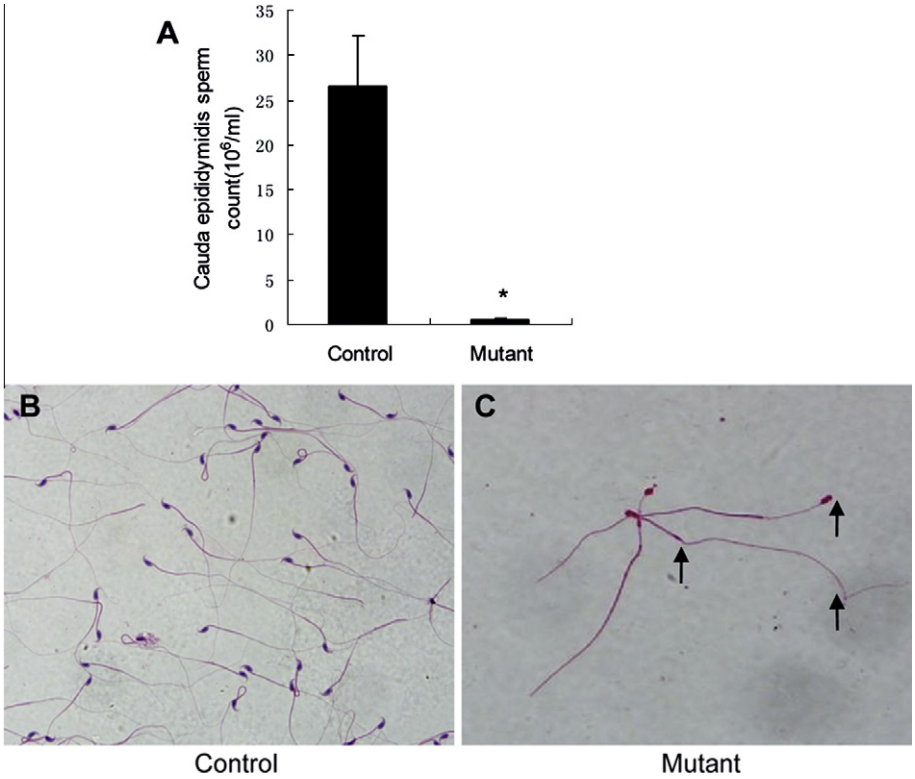

Fig. 3. *Vasa-cre;Dicer1*$^{flox/flox}$ (mutant) mice are deficient in generating normal sperm. Sperm from cauda epididymides of adult mutant mice were counted using a hemocytometer (A) and H&E stained (B and C), which showed a large decrease in number and abnormalities in morphology and structure (arrows in C).

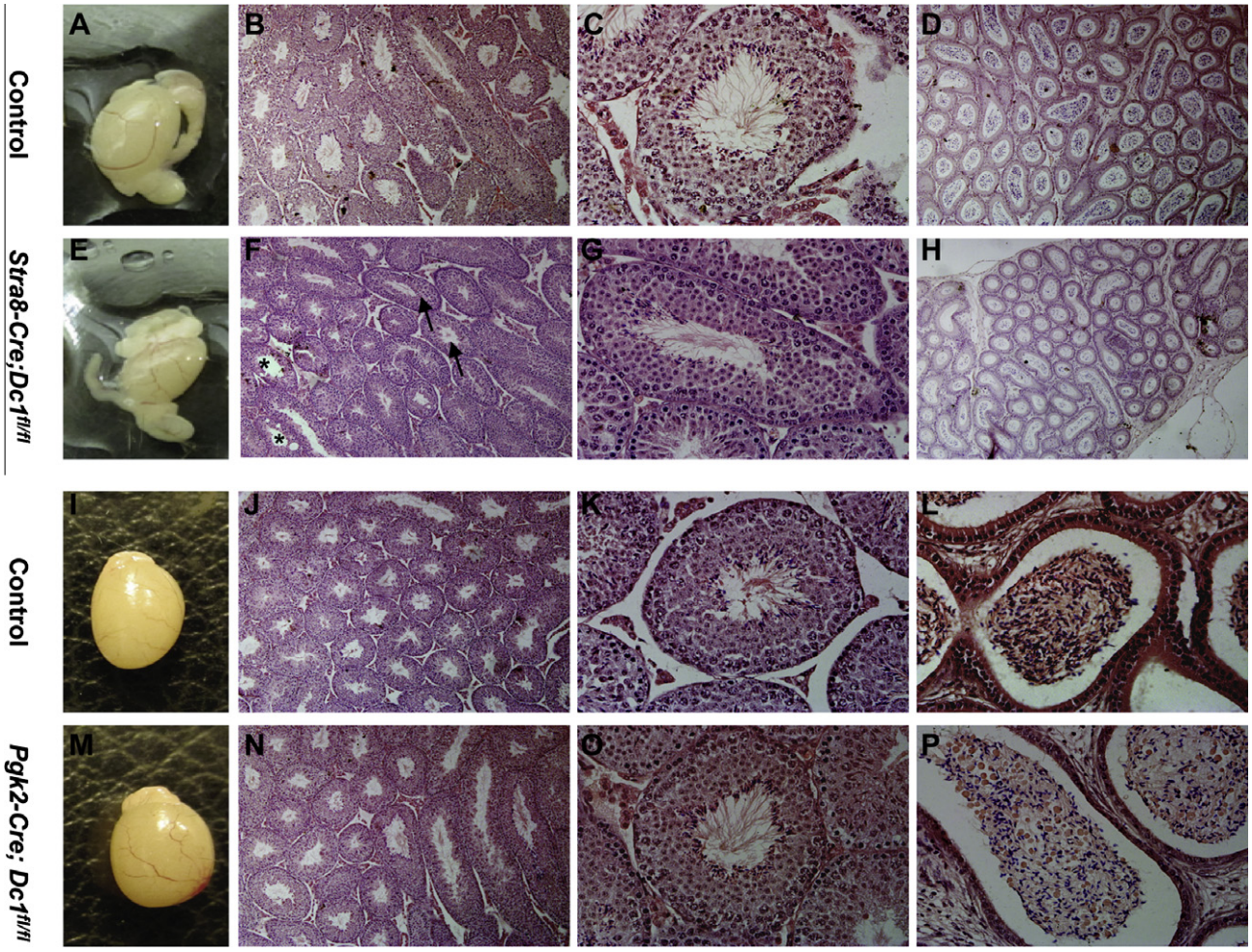

Fig. 4. Different phenotypes emerge when *Dicer1* is inactivated at the spermatogonia or spermatocyte stage. *Stra8-cre;Dicer1*$^{flox/flox}$ mutant mice showed reduction in testes size (E) and lack of elongated spermatozoa and vacuolation in some seminiferous tubules (∗ in F), but elongated spermatozoa could still be observed by the naked eye in many tubules (arrows in F and O) and epididymides (H). No distinct alterations were observed in testes size or H&E stained testes sections of *Pgk2-cre; Dicer1*$^{flox/flox}$ male mice (M–O), except for exfoliated cells present in the epididymis (P).

Table 2
Stra8-cre; Dicer$^{flox/flox}$ male mice are subfertile.

Male	Vaginal plugs	Litters	Pups
Mutant 1	1	1	2
Control 1	1	1	9
Mutant 2	1	1	1
Control 2	1	1	10
Mutant 3	1	0	0
Control 3	1	1	10

abnormalities together may partially explain the sterility of *Dicer1* mutant mice.

3.6. Ablation of Dicer1 in other stages of spermatogenesis results in several different phenotypes

We also generated two more *Dicer1* deficient mice using *Stra8-cre* (expressing *Cre*-recombinase from P3 in testis) and *Pgk2-cre* (expressing *Cre*-recombinase only in spermatocytes and spermatids) transgenic mice[27,28].

The testes of *Stra8-cre;Dicer1*$^{flox/flox}$ males at P60 displayed a nearly 50% reduction in size compared to control littermates. Histological analysis revealed several defects, including oligoospermia and vacuolization, in seminiferous tubules of mutant mice, which were also found in *Vasa-cre;Dicer1*$^{flox/flox}$ mice described above. However, the phenotype of *Stra8-cre;Dicer1*$^{flox/flox}$ mice displayed here was slightly milder than that of the *Vasa-cre;Dicer1*$^{flox/flox}$ mice. A small number of normal tubules were still present in testis, and elongated spermatozoa were not absent from the epididymides (Fig. 4A–H). More significantly, *Stra8-cre;Dicer1*$^{flox/flox}$ males were subfertile, in contrast to the sterility of *Vasa-cre;Dicer1*$^{flox/flox}$ males (Table 2). As for *Pgk2-cre;Dicer1*$^{flox/flox}$ mice, no distinguishable phenotype could be observed when compared to control littermates, except for a large amount of exfoliated cells in the epididymides (Fig. 4I–P). These results indicated that the severity of phenotypes in *Dicer1* mutant testes was dependent on the stages at which *Dicer1* had been inactivated.

4. Discussion

It is widely recognized that miRNAs play critical roles in many highly regulated processes. It was previously reported that the expression pattern of miRNA in the testis varies temporally and spatially [29]. Based on these facts, we speculated that miRNAs are indispensible for maturation of germ cells. To investigate whether the global loss of mature miRNAs would cause several defects in spermatogenesis, we specifically eliminated *Dicer1*, which is vital to miRNA biogenesis, in germ cells. Not surprisingly,

we found that the phenotype of *Dicer1* mutant mice was abnormality in spermatogenesis and fertility.

In fact, the function of *Dicer1* in the germline has been evaluated by two groups using *TNAP-cre* transgenic mice. However, the reliability and accuracy of their research was compromised by the ectopic and low-efficient expression of *Cre*-recombinase driven by the *TNAP* promoter. Therefore, it was necessary to use a specific, fully penetrant *cre* line (such as *Vasa-cre* here) in order to better study the function of *Dicer1* in spermatogenesis. In our work here, we initially compared the phenotype between *Dicer1* mutant mice (*Vasa-cre;Dicer1*$^{flox/flox}$) and wild-type littermates at P40. The histological analysis demonstrated that most of the seminiferous tubules and epididymis of mutant mice lacked elongated spermatozoa, and several of the tubules showed severe vacuolization and disorder. These observations together with the finding that a great number of round spermatids existed in the tubules suggested that spermatogenesis may be mostly arrested at the stage of round spermatids in the mutant mice.

Quantitative real-time PCR and flow cytometry assays were performed to illustrate the proportion of haploid cells in testes. We found that the proportion of haploid cells was largely decreased in the mutant mice testis at P40. This result may easily be explained by the increase of apoptosis germ cells detected by the TUNEL assay and the decrease in proportion of spermatogonia confirmed by the quantitative real-time PCR of *Pou5f1*, which could have subsequently caused the reduced number of haploid cells after completion of the first wave of spermatogenesis at P40. Surprisingly, when we examined the proportion of haploid cells in adult mutant mice (4 months), no significant change was observed compared to controls. We believe that in the mutant adult mice, newly developed haploid cells may have compensated for the decreased haploid cell number after several waves of spermatogenesis. Interestingly, these findings, in turn, provided new evidence that the *Dicer1* deficient germ cells were primarily blocked at the stage of round spermatids and not at the other stages of spermatogenesis.

Spermatogenesis is a highly regulated multi-step process. In the present study, deletion of *Dicer1* as early as at the prospermatogonia stage did not lead to the complete arrest of spermatogenesis at any specific stage. Initially, despite the decrease in number (confirmed by the decreased *Pou5f1* transcript level), spermatogonia were capable of differentiating into spermatocytes, which underwent certain events of meiosis and subsequently developed into haploid germ cells (round spermtids). Although most germ cells were arrested at the stage of round spermatids, a small number could still be transformed to form elongated spermatozoa. Unfortunately, approximately 80% of the elongated spermatozoa in mutant mice were morphologically abnormal, which may have ultimately caused the inability to generate offspring. This complex phenotype gave rise to the question of why the same type of germ cells lacking *Dicer1* did not follow the same differentiation destiny. It is also unclear whether the spermatogonia, spermatocytes and the haploid round spermatids in the mutant testis possessed the identical transcript or expression patterns as in the control testes. In other words, without further analysis, we cannot exclude the possibility that the loss of *Dicer1* would lead the germ cells to accumulate defects at different stages of spermatogenesis, which would culminate in the failure to generate adequately functional sperm. If so, it is safe to make the assumption that if *Dicer1* was inactivated in the later stages of spermatogenesis, the phenotype would be milder than that of *Vasa-cre;Dicer1*$^{flox/flox}$ mice. The *Stra8-cre;Dicer1*$^{flox/flox}$ and *Pgk2-cre;Dicer1*$^{flox/flox}$ mice were obtained to experimentally prove our assumption. Luckily, as expected, we observed a milder phenotype in *Stra8-cre;Dicer1*$^{flox/flox}$ males, in which *Dicer1* was inactivated as early as the spermatogonia stage, and even no obvious changes were seen in the phenotype of *Pgk2-cre;Dicer1*$^{flox/flox}$ males, in which *Dicer1* was inactivated as early as the spermatocyte stage.

Our study was carried out on the basic presumption that loss of *Dicer1* would affect spermatogenesis due to the interruption of miRNA biogenesis. However, other critical functions of *Dicer1* should not be ignored. For instance, overexpression of *Dicer1* has been associated with tumorgenesis [30–32], indicating that Dicer1 plays a role in regulation of cell proliferation, which is independent of the miRNA biogenesis pathway to some extent. It has also been reported that *Dicer1* is one of the components of the chromatid body (CB) in round spermatids, and the aberrant transformation of CB leads to morphological defects in elongated spermatozoa [33,34]. Therefore, it is unclear whether the absence of *Dicer1* in CB would interfere with its transformation and subsequently cause the morphological abnormality of elongated spermatozoa we observed. Further investigations are required to determine the mechanisms underlying the observed defects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.118.

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