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MicroRNA-382 inhibits the proliferation of mouse spermatogonia by targeting *Kmt5a*

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Abstract: Spermatogenesis is a highly efficient and intricate process in the testis by which mature spermatozoa are produced daily to maintain lifelong male fertility. Essential to this process are spermatogonia capable of both proliferation and differentiation. Nevertheless, the underlying mechanisms for spermatogonial proliferation and differentiation remain poorly understood. MicroRNAs (miRNAs) are a category of non-coding small RNAs with regulatory functions by binding to the 3' untranslated region (UTR) of the target mRNA. Previous studies have demonstrated that miRNAs are capable of modulating cell proliferation, differentiation and apoptosis, but the roles of individual miRNAs in spermatogonial fate determination remain largely elusive. Here, by using a mouse spermatogonial cell line (GC-1), we investigated the role for miRNA-382 in spermatogonial proliferation. We found that pre-miRNA-382 was expressed in spermatogonia. The luciferase reporter assay demonstrated *Kmt5a* but not *Top1* as a target gene of miRNA-382. Overexpression of miRNA-382 by transfecting a miRNA mimic downregulated *Kmt5a* at both RNA and protein levels, and further reduced the proliferation and viability of spermatogonia. Knockdown of *Kmt5a* by RNA interference (RNAi) resulted in a uniform phenotype in spermatogonia. We therefore conclude that miRNA-382 inhibits the proliferation of mouse spermatogonia by targeting *Kmt5a*. Our finding extends the knowledge about the regulatory roles of miRNAs in spermatogonia and lays the groundwork for diagnosis and treatment of male infertility.

Introduction

Spermatogenesis is a highly orchestrated and efficient process in the testis by which infinite spermatozoa are produced daily to maintain lifelong male fertility. Essential to this process are spermatogonia capable of both proliferation and differentiation. The balance between spermatogonial proliferation and differentiation is crucial and disturbance of this balance, i.e., excessive proliferation or differentiation, can lead to tumor-like germ cell clusters or germ cell depletion, respectively, and both will result in azoospermia ultimately (Silber, 2000). Spermatogonia can be divided into undifferentiated and differentiating spermatogonia. Undifferentiated spermatogonia consist of spermatogonial stem cells (SSCs) and progenitors committed to differentiation, whereas differentiating spermatogonia later develop to spermatocytes that generate haploid spermatids via meiosis (Jan *et al.*, 2012). Being the cornerstone of spermatogenesis, spermatogonia locate at the basement membrane of seminiferous tubules in the testis, and their behaviors are stringently modulated by a variety of growth factors secreted by the adjacent somatic cells. Of these, glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF), both secreted by Sertoli cells, play pivotal roles in the proliferation of spermatogonia (Kanatsu-Shinohara and Shinohara, 2013; Makela and Hobbs, 2019). Despite this, the underlying mechanisms for spermatogonial proliferation remain largely elusive.

MicroRNAs (miRNAs) are a category of non-coding small RNAs (~22 nt) with regulatory functions. By binding to the 3' untranslated region (UTR) of the target mRNA, miRNAs are able to degrade mRNA or repress translation, thereby regulating the expression of target genes (Thomas *et al.*, 2010). MiRNAs are generally expressed in rodent testes (Yan *et al.*, 2007), and several miRNAs/miRNA clusters have been reported to be involved in the regulation of SSC maintenance and spermatogenesis in mammals (Wang and Xu, 2015; Chen *et al.*, 2017b). For instance, miRNA-21 was highly expressed in THY1^{+} mouse undifferentiated spermatogonia, and it was regulated by ETV5, a downstream target of GDNF signaling essential for SSC maintenance. Downregulation of miRNA-21 led to spermatogenic cell apoptosis and a reduction of SSC number

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(Niu *et al.*, 2011). MiRNA-17-92 and miRNA-106b-25 were downregulated by retinoic acid (RA) treatment in THY1$^+$ cells, suggesting their roles in SSC self-renewal (Tong *et al.*, 2012). By repressing *c-Kit*, miRNA-221/222 was able to maintain the undifferentiated state of SSCs (Yang *et al.*, 2013). Similarly, miRNA-20 and miRNA-160a, both originated from the miRNA-17-92 cluster, played important roles in SSC self-renewal, possibly by downregulating spermatogonial differentiation factor *Stat3* (He *et al.*, 2013). A recent article also reported that miRNA-100 stimulated SSC proliferation via targeting *Stat3* (Huang *et al.*, 2017).

It has been reported that around 1,000 miRNAs are encoded in the mouse genome. Thus, more miRNAs are expected to function in spermatogonial proliferation (He *et al.*, 2013). Previous studies showed that miRNA-382 could suppress cancer and tumorigenesis (Xu *et al.*, 2014; Xu *et al.*, 2015). Nevertheless, its role in spermatogonia has so far not been reported. In the present study, by using a mouse spermatogonial cell line (GC-1) and a miRNA mimic to overexpress the miRNA, we found that miRNA-382 inhibited the proliferation of mouse spermatogonia by targeting *Kmt5a*. Our finding extends the knowledge about the regulatory roles of miRNAs in spermatogonia and lays the groundwork for diagnosis and treatment of male infertility.

Materials and Methods

Cell culture

The GC-1 mouse type B spermatogonial cell line (Hofmann *et al.*, 1992) and Hela cell line were cultured in a medium consisting of DMEM (high glucose, Sigma) supplemented with 10% fetal bovine serum (FBS, Thermofisher), 1% penicillin/streptomycin (Thermofisher) and 1% non-essential amino acid (NEAA, Thermofisher). The cells were refreshed every 2–3 days and maintained at 37°C in an atmosphere of 5% CO_2 in air.

Reverse transcription-PCR (RT-PCR) and quantitative real-time PCR (q-PCR)

Total RNAs were extracted from cells with the Trizol reagent (Thermofisher), following the manufacturer's protocol. After reverse transcription by using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China), cDNA samples were subjected to PCR amplification. The primer information is as follows:
pre-miRNA-382-forward: 5'-AAGTTGTTCGTGGTGGATTCG-3'
and -reverse: 5'-GTGCAGGGTCCGAGGTATTC-3';
U6-forward: 5'-CTCGCTTCGGCAGCACA-3'
and -reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

The q-PCR reaction was performed in a 20 µL volume system including 10 µL SYBR Green II PCR Mix (Takara) by using an IQ5 (Bio-Rad, Berkeley, CA, USA). q-PCR reactions were run in triplicates of three independent experiments. *Gapdh* was used as a reference gene, and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Taylor *et al.*, 2019). The primer information is as follows:
Kmt5a-forward: 5'-TTCACTGATGCCAAGAAGCGG-3'
and -reverse: 5'-TAGGCGGTTCGTTTCTTGAGTG-3';
Gapdh-forward: 5'-AAGGGCTCATGACCACAGTC-3'
and -reverse: 5'-ACACATTGGGGGTAGGAACA-3'.

Luciferase reporter assay

The 3' UTR (containing the predicted miRNA-382-binding site) or mutated 3' UTR (containing 4–6 nt of mutation at the predicted miRNA-382-binding site) of *Kmt5a* (NCBI accession number: NM_030241.3) or *Top1* (NCBI accession number: NM_009408.2) was cloned into psiCHECK-2 Dual-Luciferase Report plasmids (Promega, Madison, WI) following standard procedures. Specifically, RNAs were extracted from cells and cDNA samples were prepared as described, and then the 3' UTR of *Kmt5a* or *Top1* was PCR-amplified, using the following primers:
Kmt5a-3' UTR-forward: 5'-GGCGTGCCTCACCTCATCCTC-3'
and -reverse: 5'-ATACAAAGCTAAGCCACAA-3';
Top1-3' UTR-forward: 5'-TCAATTATCTGGACCCTAGG-3'
and -reverse: 5'-AACTAACAATGCCTCTAAA-3'.

To prepare the psiCHECK2-*Kmt5a*-3' UTR-mut or psiCHECK2-*Top1*-3' UTR-mut plasmids, mutated PCR products were generated using the following primers:
Kmt5a-3' UTR-mut-1-forward: 5'-GGCGTGCCTCACCTCA-TCCTC-3'
and -1-reverse: 5'-CCTATATACACGTTGCTCGCTTTTCT-GTGGGTT-3';
Kmt5a-3' UTR-mut-2-forward: 5'-CCCACAGAAAAGCGA-GCAACGTGTATATAGGT-3'
and -2-reverse: 5'-ATACAAAGCTAAGCCACAA-3';
Top1-3' UTR-mut-1-forward: 5'-TCAATTATCTGGACCCTAGG-3'
and -1-reverse: 5'-TTTAAATAACAACTCCATAGGACAACT-3';
Top1-3' UTR-mut-2-forward: 5'-GTTGTCCTATGGAGTTG-TTATTTAA-3'
and -2-reverse: 5'-AACTAACAATGCCTCTAAA-3'.

The mutated PCR products were then recombined by way of overlap PCR. Later, the empty psiCHECK2 vector was subjected to double enzyme digestion, using QuickCut^{TM}Not I and QuickCut^{TM}Xho I (Takara, Japan). The linearized plasmids were ligated with the gel-extracted PCR products using the T4 DNA ligase (Takara, Japan). The recombinant plasmids were amplified following standard procedures, and the correct insertion was confirmed by repeating double-enzyme digestion and Sanger-sequencing.

For the luciferase reporter assay, Hela cells were transfected with the constructed psiCHECK2-*Kmt5a*-3' UTR, psiCHECK2-*Kmt5a*-3' UTR-mut, psiCHECK2-*Top1*-3' UTR or psiCHECK2-*Top1*-3' UTR-mut plasmids, in combination with a miRNA-382 mimic or negative control, by the Lipofectamine 2000 transfection reagent (Thermofisher). Cells were lysed 24 hours after transfection, and then the relative luciferase activity was measured according to the protocol provided by the manufacturer.

MiRNA-382 mimic treatment

The miRNA-382 mimic and negative control were purchased from Genepharma Company (Shanghai, China). Transfection was performed by using the Lipofectamine 2000 transfection reagent (Thermofisher), following the protocol provided by the manufacturer. Two days after transfection, cells were harvested for downstream experiments.

Kmt5a siRNA treatment

The *Kmt5a* siRNA and negative control were purchased from Genepharma Company (Shanghai, China). GC-1 spermatogonia were transfected with the Lipofectamine 2000 transfection

reagent (Thermofisher), following the protocol provided by the manufacturer. Two days after transfection, cells were harvested for downstream experiments. The sequence information of the siRNAs is as follows:
against *Kmt5a*:
5'-CCGGGGAATCTACAGGAAGCGAGAATCAAGAGTTC-TCGCTTCCTGTAGATTCCTTTTTG-3';
the negative control:
5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGG-TGCTCTTCATCTTGTTGTTTTTG-3'.

Western blot
Cells were lysed with the RIPA buffer, and then the proteins were extracted and quantified. Denatured proteins were separated by SDS-PAGE, and then transferred to the PVDF membrane. The blot was incubated with primary antibodies (rabbit anti-KMT5A, Proteintech, 1:2,000; mouse anti-GAPDH, Santa Cruz, 1:1,000) at 4°C overnight. After washing on the next day, the blot was incubated with the horseradish peroxidase-conjugated anti-rabbit or -mouse secondary antibody (Abcam, Cambridge, UK, 1:10,000) for 2 hours at room temperature. Protein signals were detected using a Bio-Rad Chemidoc XRS with a Western Bright ECL Kit (Advansta, Menlo Park, CA, USA). The protein band density was analyzed by Image-Pro Plus (Media Cybernetics, USA). To quantify the protein level of KMT5A, the KMT5A band density was divided by that of GAPDH.

EdU cell proliferation assay
GC-1 spermatogonia transfected with a miRNA-382 mimic, *Kmt5a* siRNA or the corresponding negative control were subjected to an EdU cell proliferation assay (Cell-Light EdU Apollo 488, ribobio, China). In brief, two days after transfection, cells were incubated with the medium containing EdU (1:1,000) for 2 hours at room temperature, and then the cells were subjected to immunocytochemistry, following the manufacturer's protocol. The ratio of cells incorporating EdU was calculated.

CCK-8 cell viability assay
GC-1 spermatogonia transfected with a miRNA-382 mimic, *Kmt5a* siRNA or the corresponding negative control were subjected to a CCK-8 cell viability assay (Vazyme Biotech Co., Ltd., China). In brief, cells were pre-seeded to 96-well plates at a density of 3,000 cells/well. Two days after transfection, cells were incubated with the medium containing CCK-8 for 2 hours. The absorbance at 450 nm was measured according to the protocol provided by the manufacturer.

Statistics
Data were presented as the mean ± standard error of the mean (SEM) of three independent experiments (n = 3). Differences between groups were assessed with the Student's *t*-test. $P < 0.05$ was considered statistically different.

Results

Pre-miRNA-382 is expressed in spermatogonia
We first used RT-PCR to detect the expression of miRNA-382 in spermatogonia. As shown in Fig. 1A, pre-miRNA-382 was expressed in GC-1 mouse spermatogonia, suggesting potential regulatory roles of miRNA-382 in spermatogonia.

Kmt5a, but not Top1, is a target gene of miRNA-382
Next, we probed the target genes of miRNA-382. By using TargetScan (Doran and Strauss, 2007), we predicted that *Kmt5a* and *Top1* could be potential target genes of miRNA-382 (Fig. 1B). To validate these predicted target genes, we performed a luciferase reporter assay. We subcloned the 3' UTR (containing the predicted miRNA-382-binding site) or mutated 3' UTR (containing 4–6 nt of mutation at the predicted miRNA-382-binding site) of *Kmt5a* (1627 bp) or *Top1* (1230 bp, Fig. 1C) into the luciferase reporter vector psiCHECK-2, and generated the psiCHECK2-*Kmt5a*-3' UTR, psiCHECK2-*Kmt5a*-3' UTR-mut, psiCHECK2-*Top1*-3' UTR and psiCHECK2-*Top1*-3' UTR-mut plasmids, respectively. Colony PCR and double-enzyme digestion followed by gel electrophoresis detection validated the recombinant plasmids (Fig. 1D). Then, we co-transfected a miRNA-382 mimic or negative control in combination with the recombinant psiCHECK2 vector into Hela cells, and detected that only the miRNA-382 mimic group co-transfected with the psiCHECK2-*Kmt5a*-3' UTR plasmids showed a decrease in the relative luciferase activity (Fig. 1E), indicating that miRNA-382 binds to the *Kmt5a* 3' UTR and that *Kmt5a*, but not *Top1*, is a direct target gene of miRNA-382.

MiRNA-382 inhibits the expression of Kmt5a in spermatogonia
Subsequently, we transfected a miRNA-382 mimic or negative control into GC-1 spermatogonia. Two days after transfection, q-PCR analysis was performed to quantify the expression of *Kmt5a*. Compared with the negative control, the mRNA level of *Kmt5a* was significantly reduced after treatment with the miRNA-382 mimic (Fig. 2A). Western blot analysis showed a consistent decrease of KMT5A at the protein level (Fig. 2B), supporting that miRNA-382 inhibits the expression of *Kmt5a* in spermatogonia.

MiRNA-382 inhibits the proliferation and viability of spermatogonia
Two days after transfection, cells treated with a miRNA-382 mimic exhibited reduced cell proliferation (Fig. 3A) in comparison with the negative control (Fig. 3B). To pinpoint that overexpression of miRNA-382 repressed spermatogonial proliferation, we conducted an EdU incorporation assay. The ratio of cells incorporating the thymidine analog EdU was significantly decreased in the miRNA-382 mimic group (Fig. 3C). We also performed a CCK-8 cell viability assay, and detected that transfection with a miRNA-382 mimic consistently reduced the spermatogonial viability (Fig. 3D). The overall results demonstrate that miRNA-382 inhibits the proliferation and viability of spermatogonia.

Knockdown of Kmt5a inhibits the proliferation of spermatogonia
To investigate whether the reduced spermatogonial proliferation and viability were resulted from the downregulation of *Kmt5a*, we depleted *Kmt5a* by siRNA transfection. The q-PCR result demonstrated the significant depletion of *Kmt5a* by siRNA treatment (Fig. 4A). As expected, the spermatogonial proliferation and viability were markedly reduced compared

FIGURE 1. Identification of the target genes for miRNA-382.
(A) Expression of pre-miRNA-382 in spermatogonia. NC: negative control (-RT). U6 is used as a reference. (B) Prediction of the target genes for miRNA-382 by TargetScan. (C) Target regions of the (mutated) 3' UTR of *Kmt5a* and *Top1* for miRNA-382. (D) (1, 5) RT-PCR amplification of the 3' UTR of *Kmt5a* (1) and *Top1* (5); (2, 6) Colony PCR amplification of the 3' UTR of *Kmt5a* (2) and *Top1* (6) from different recombinants; (3, 7) Gel electrophoresis detection of the recombinant psiCHECK2-*Kmt5a*-3' UTR (3) and psiCHECK2-*Top1*-3' UTR (7) after double-enzyme digestion; (4, 8) Gel electrophoresis detection of the recombinant psiCHECK2-*Kmt5a*-3' UTR-mut (4) and psiCHECK2-*Top1*-3' UTR-mut (8) after double-enzyme digestion. (E) The luciferase reporter assay. Hela cells were co-transfected with a miRNA-382 mimic or negative control in combination with the recombinant psiCHECK2 plasmids. psiCHECK2: the empty vector transfection group. Data are presented as the mean ± SEM, n = 3. *: $P < 0.05$.

FIGURE 2. Overexpression of miRNA-382 downregulates *Kmt5a*.
(A) Expression of *Kmt5a* in spermatogonia after transfection with a miRNA-382 mimic or negative control. (B) Western blot analysis of KMT5A and the KMT5A band intensities in relation to GAPDH. GAPDH is used as a loading control. Data are presented as the mean ± SEM, n = 3. *: $P < 0.05$.

with the negative control, as shown by the EdU (Fig. 4B) and CCK-8 assay (Fig. 4C), respectively. Taken together, we conclude that miRNA-382 inhibits the proliferation of mouse spermatogonia by targeting *Kmt5a* (Fig. 4D).

Discussion

MiRNA-382 has long been studied and has been shown to play divergent roles in a variety of tumor cell categories. Xu and colleagues (Xu *et al.*, 2014) found that miR-382 could suppress tumor growth and improve chemo-sensitivity in osteosarcoma by targeting *Klf12* and *Hip3*. The same group later reported that miR-382 repressed osteosarcoma metastasis and relapse (Xu *et al.*, 2015). In ovarian cancer cells, miR-382 suppressed the migration and invasion by targeting *Ror1* (Tan *et al.*, 2016), and in colorectal cancer cells, miRNA-382 repressed the cellular growth and invasion (Zhou *et al.*, 2016). Also, miRNA-382 played similar roles in

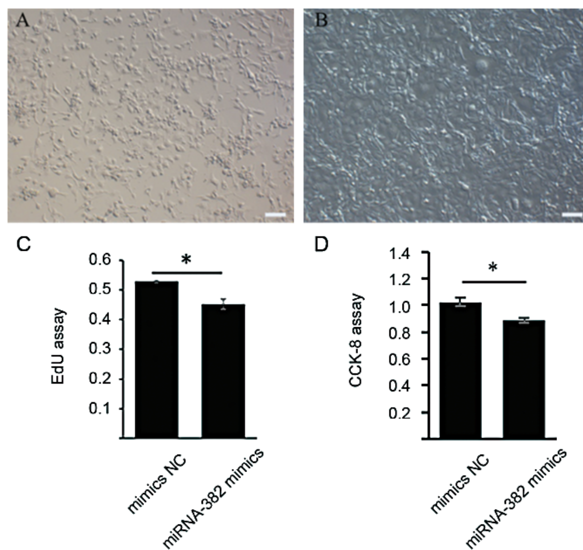

FIGURE 3. Overexpression of miRNA-382 inhibits the proliferation and viability of spermatogonia.
(A, B) Representative images of GC-1 spermatogonia 2 days after transfection with a miRNA-382 mimic (A) or negative control (B). Bar = 100 μm. (C) The ratio of cells incorporating the thymidine analog EdU. (D) The relative cell viability detected by the CCK-8 assay. Data are presented as the mean ± SEM, n = 3. *: $P < 0.05$.

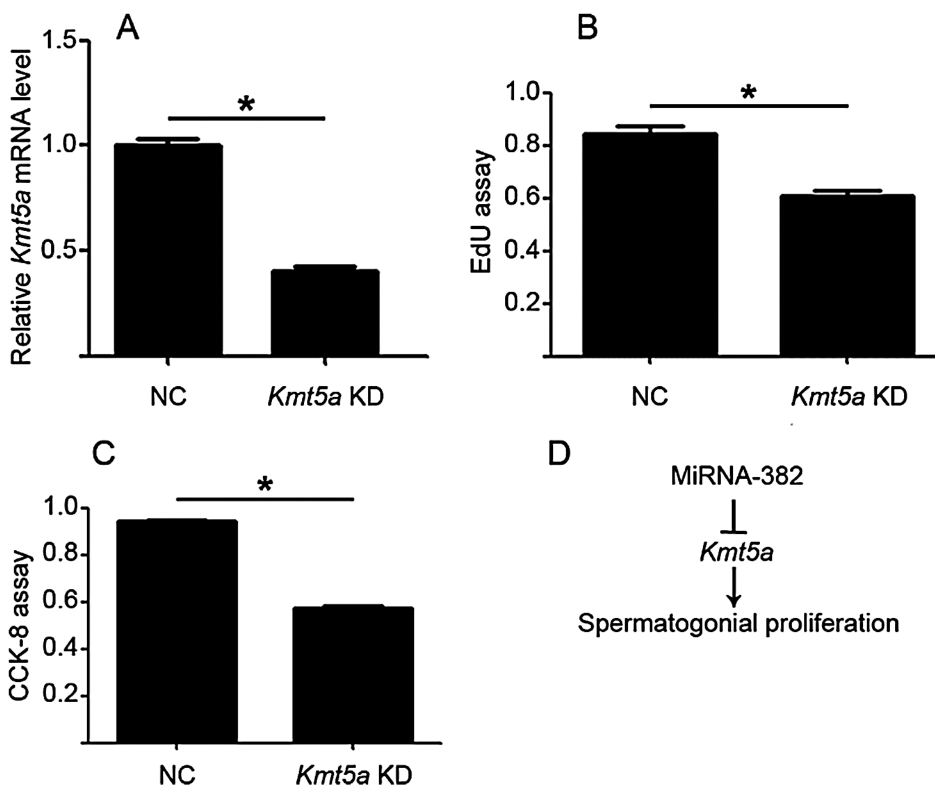

FIGURE 4. Knockdown of *Kmt5a* inhibits the proliferation of spermatogonia.
(A) Expression of *Kmt5a* in spermatogonia after transfection with a *Kmt5a* siRNA or negative control. (B) The ratio of cells incorporating the thymidine analog EdU. (C) The relative cell viability detected by the CCK-8 assay. Data are presented as the mean ± SEM, n = 3. *: $P < 0.05$. (D) A schematic overview illustrating the regulatory mechanism for miRNA-382 in spermatogonial proliferation.

non-small cell lung cancer cells, characterized by its inhibitory role in the tumor progression (Chen *et al.*, 2017a). Conversely, miRNA-382 promoted the survival and viability of breast cancer cells, thereby exacerbating tumorigenesis and metastasis (Ho *et al.*, 2017). Hence, previous studies have well demonstrated that miRNA-382 is related to cell proliferation. Here, by transfecting a miRNA mimic, we have for the first time shown the inhibitory role of miRNA-382 in spermatogonial proliferation.

In the present study, we found that miRNA-382 only bond to the 3' UTR of *Kmt5a*, indicating that *Kmt5a*, but not *Top1*, is a direct target gene of miRNA-382, which was later validated by the downregulation of *Kmt5a* at both RNA and protein levels by overexpression of miRNA-382 in spermatogonia. KMT5A, also known as PR-Set7 or SETD8, is involved in H4K20 methylation and DNA transcription (Kapoor-Vazirani *et al.*, 2011). KMT5A can also bind to proliferating cell nuclear antigen (PCNA) and modulate the initiation of DNA replication (Tardat *et al.*, 2010). In addition, KMT5A has been reported to be implicated in mitosis, and its degradation is indispensable for the correct cell cycle progression (Stukenberg *et al.*, 1997; Wu *et al.*, 2010). Here, by way of EdU and CCK-8 assays, we showed that downregulation of *Kmt5a* repressed spermatogonial proliferation, consistent with previous studies in several tumor cell types. Future studies should focus on how KMT5A facilitates spermatogonial proliferation, and whether this process involves DNA replication or DNA methylation regulation. In addition, KMT5A may also be involved in spermatogonial differentiation, which warrants future exploration. Unraveling the expression profiles of KMT5A in different spermatogenic cell types would be a requisite in both respects.

Our study, which has for the first time uncovered that miRNA-382 inhibits the proliferation of mouse spermatogonia by targeting *Kmt5a*, contributes to the knowledge about the

regulatory roles of miRNAs in spermatogonia and spermatogenesis. This regulatory mechanism of miRNA-382, although gained via our preceding in vitro study, is highly likely to be translatable to the *in vivo* scenario. Abnormal expression of miRNA-382 in spermatogonia may disturb the balance between SSC self-renewal and differentiation, further resulting in male infertility. Given this, the need for in vivo phenotypic analyses remains, and generation of a (conditional) knockout mouse model for miRNA-382 would be of utmost help in this regard.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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