RNA helicase DDX5 acts as a critical regulator for survival of neonatal mouse gonocytes

Qing Xia1,2,3,4,5 | Guizhong Cui3 | Ye Fan6 | Xiuqin Wang1,2,3,4 | Gongcheng Hu1,2,3,4 |
| Lisha Wang1,2,3,4 | Xi Luo1,2,3,4 | Lele Yang1,2,3,4 | Qingqing Cai1 | Kaibiao Xu1,2,3,4 |
| Wenjing Guo1 | Minghui Gao1 | Yingying Li3 | Ji Wu7 | Wei Li8 | Jiayu Chen9 |
| Huayu Qi1,2,3,5 | Guangdun Peng1,2,3,4,5 | Hongjie Yao1,2,3,4 |

1CAS Key Laboratory of Regenerative Biology and Guangdong Provincial Key Laboratory of Stem Cell and Regenerative Medicine, Joint School of Life Sciences, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou Medical University, Guangzhou, China
2GIBH-HKU Guangdong-Hong Kong Stem Cell and Regenerative Medicine Research Centre, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China
3Biland Laboratory (Guangzhou Regenerative Medicine and Health GuangDong Laboratory), Guangzhou, China
4Institute of Stem Cell and Regeneration, Chinese Academy of Sciences, Beijing, China
5University of Chinese Academy of Sciences, Beijing, China
6Department of Respiratory Disease, Xinqiao Hospital, Third Military Medical University, Chongqing, China
7Bio-X Institutes, Shanghai Jiao Tong University, Shanghai, China
8State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China
9Clinical and Translation Research Center of Shanghai First Maternity and Infant Hospital, Shanghai Key Laboratory of Signaling and Disease Research, School of Life Sciences and Technology, Tongji University, Shanghai, China

Abstract

Objectives: Mammalian spermatogenesis is a biological process of male gamete formation. Gonocytes are the only precursors of spermatogonial stem cells (SSCs) which develop into mature spermatozoa. DDX5 is one of DEAD-box RNA helicases and expresses in male germ cells, suggesting that Ddx5 plays important functions during spermatogenesis. Here, we explore the functions of Ddx5 in regulating the specification of gonocytes.

Materials and Methods: Germ cell-specific Ddx5 knockout (Ddx5−/−) mice were generated. The morphology of testes and epididymides and fertility in both wild-type and Ddx5−/− mice were analysed. Single-cell RNA sequencing (scRNA-seq) was used to profile the transcriptome in testes from wild-type and Ddx5−/− mice at postnatal day (P) 2. Dysregulated genes were validated by single-cell qRT-PCR and immunofluorescent staining.

Results: In male mice, Ddx5 was expressed in germ cells at different stages of development. Germ cell-specific Ddx5 knockout adult male mice were sterile due to completely devoid of germ cells. Male germ cells gradually disappeared in Ddx5−/− mice.
from E18.5 to P6. Single-cell transcriptome analysis showed that genes involved in cell cycle and glial cell line-derived neurotrophic factor (GDNF) pathway were significantly decreased in Ddx5-deficient gonocytes. Notably, Ddx5 ablation impeded the proliferation of gonocytes.

**Conclusions:** Our study reveals the critical roles of Ddx5 in fate determination of gonocytes, offering a novel insight into the pathogenesis of male sterility.

---

## 1 | INTRODUCTION

In mice, as the foundation for the continuity of spermatogenesis, SSCs are derived from gonocytes that are originated from primordial germ cells (PGCs) residing in the proximal epiblast during gastrulation. PGCs migrate to the developing gonad and differentiate into male or female germ cells following sex determination. In the fetal male gonads, germ cells are referred to as gonocytes. Testicular gonocytes stay in the G0/G1 phase of cell cycle from embryonic day (E) 13.5. The quiescent gonocytes begin to relocate to the periphery from the central region of seminiferous tubules, and resume mitotic division shortly after birth. Gonocytes give rise to undifferentiated spermatogonia or differentiating spermatogonia through gonocytes-to-spermatogonia transition (GST), which is the gateway to spermatogenesis in neonatal testes. Proliferation and migration of gonocytes are two crucial events that take place between P0 and P6. Gonocytes and spermatogonia are closely related, but they have different morphology, transcriptome, DNA methylation and chromosome architecture.

Although tremendous efforts have been focused on studying the maintenance and differentiation of SSCs, the formation of SSCs (especially the proliferation, migration and transition of gonocytes), is still unclear. Sertoli cells have been reported to play important roles in regulating these processes. Platelet-derived growth factor (PDGF), synthesized and secreted by Sertoli cells, has the remarkable capability to drive the survival and migration of the gonocytes. As a RNA-binding protein, DD5 has been shown to be involved in multiple biological processes. Ddx5 has been reported to express in male germline and plays a negative role in WNT signaling regulation of the GC-1 spg cell. Disruption of Ddx5 in adult spermatogonia results in infertility. Although it is clear that Ddx5 is required for the maintenance of spermatogonia in adult testis, we do not yet know whether it is required for the formation of the spermatogonia.

In this study, we found that Ddx5 was highly expressed in male germ cells from prenatal period. We generated germ cell-specific Ddx5 knockout mice by using transgenic mice expressing Cre recombinase driven by the Mvh promoter (Mvh-Cre) and found that Ddx5-/- mice showed significantly smaller and lighter testes than wild-type (WT) and heterozygous (Ddx5+/-) mice. Furthermore, Ddx5 knockout resulted in a complete loss of germ cells, containing only Sertoli cells within 6 days after birth, and led to azoospermia and infertility in male adults. In addition, scRNA-seq experiments indicated that Ddx5 knockout resulted in aberrant expression of genes that were associated with cell cycle and GDNF pathway in gonocytes. Interestingly, we further found that Ddx5 knockout induced dysregulation of GDNF signaling pathway and then impeded proliferation of gonocytes and gonocytes-to-spermatogonia transition.

## 2 | MATERIALS AND METHODS

### 2.1 | Generation of Ddx5 conditional knockout mice

The Ddx5 floxed mice were crossed with Mvh-Cre transgenic mice to obtain Ddx5+/- mice. Homozygous Ddx5+/- mice were obtained by crossing Ddx5+/- males and Ddx5+/- females.

### 2.2 | Assessment of fertility and fecundity

To assess fertility and fecundity, one 3-month-old male mouse of either wild-type, Ddx5+/- or Ddx5-/- was placed into a cage with a wild-type female. Cages were monitored daily. The females were checked for the presence of vaginal plugs and pregnancy. The numbers of pups born in each mating set were recorded.

### 2.3 | Immunofluorescent staining of the frozen sections

Tissues were dissected from mice immediately after euthanasia and fixed in 4% paraformaldehyde (PFA) (Beyotime, P0099) overnight at 4°C. Samples were embedded with tissue freezing medium (Leica, 020108926) and sectioned (8 μm in thickness). The sections were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, T8787) in PBS for 20 minutes and blocked with 5% BSA (Gold Bio, A-420) in PBS for 1 hour at room temperature. The sections were incubated...
with the diluted primary antibody and subsequently secondary antibody. Primary antibodies used in this study are listed in Table S2. Immunoglobulin G (IgG) was used as a negative control for the primary antibody. A final concentration of 1 mg/mL DAPI (Beyotime, C1002) was included to stain nuclei. Fluorescent images were captured with a confocal microscope (LSM800, Carl Zeiss). Images were further processed with ZEN-2012SP2-blue software. Numbers of gonocytes and the area corresponding to testis cross-section were measured using ImageJ software. To minimize the difference caused by different testicular positions, the gonocytes were shown as unit area (mm²).

2.4 RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from mouse testes using TRIzol reagent (MRC, TR118) in accordance with the manufacturer’s instructions. Nucleic acid quantification and purity are listed in Table S4. Briefly, 1 μg of total RNA was reverse-transcribed with HiScript II Q RT SuperMix for qPCR with gDNA Wiper (Vazyme, R223-01). Quantitative RT-PCR was performed using a CFX96 Real-Time System (Bio-Rad) and SYBR Green qPCR Mix (GenStar, A301-01). The PCR cycling conditions were as follows: 40 cycles of 95°C for 10 seconds (denaturation), 60°C for 10 seconds (annealing) and 72°C for 20 seconds (elongation); Expression levels were normalized to the geometric mean of Gapdh and Actin. The relative expression level of candidate genes was calculated using the formula 2^ΔΔCT as described. The primers used are listed in Table S1. All experiments were repeated three times.

2.5 Isolation of single testicular cells by fluorescence-activated cell sorting (FACS), single-cell reverse transcription and amplification

The testes at P2 were collected, de-capsulated and digested with TrypLE™ Express Enzyme (Thermo Fisher, 12605010). The cell suspensions were filtered through a 40 μm cell strainer (Corning, 22363547), and cells were collected by centrifugation at 300 x g for 5 minutes. The pellets were resuspended in DPBS (HyClone, SH30028) with 0.04% BSA and isolated with a MoFlo Astrios EQ high speed cell sorter (Beckman Coulter). Then, the cells were sorted into cell lysis buffer containing 0.45% (vol/vol) NP40 (Roche, 11332473001), followed by reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen, 18064-014) and whole transcription amplification using KAPA HiFi HotStart Ready Mix (KAPA Biosystems, KK2602).

2.6 10 × genomics single-cell sample processing and cDNA library preparation

Single testicular cells of testes at P2 were dissociated with TrypLE™ Express Enzyme (Thermo Fisher, 12605010). Cell viability was assessed by trypan blue staining. Cell capturing, cDNA amplification and library preparation were carried by Chromium Single Cell 3’ GEM, Library & Gel Bead Kit v3 (10 × Genomics, 1000075) according to the manufacturer’s instructions. The resultant libraries were size selected, pooled and sequenced using 2 × 150 paired-end sequencing protocol on an Illumina NovaSeq 6000 instrument.

3 RESULTS

3.1 Ddx5 expression during testicular development

To explore the roles of Ddx5 during male germ cell development, Ddx5 expression was evaluated in testes of postnatal mice, collect- ing every 5 days after birth according to the developmental process of spermatogenic cells. The mRNA level of Ddx5 was increased accompanying mouse age, with more prominent expression at P20 and P25, similar to that of Mvh, a germ cell-specific marker (Figure 1A,B). Moreover, we performed western blotting for DDX5 and different markers of germ cells, respectively. As expected, DDX5 protein level was gradually increased from P0 to P90, and increasing levels as spermatocytes and round spermatids developed (Figure 1C). Western blotting of spermatogenic cells isolated using unit-gravity sedimentation indicated that DDX5 was predominantly expressed in spermatocytes and round spermatids, but not in elongating spermatids (Figure 1D).

We further examined the expression and subcellular localization of DDX5 during earlier male germ lineage development by performing immunofluorescent staining (Figure 1E). Transgenic mice which express the Oct4 promoter-driven GFP (hereafter referred to as Oct4-GFP) were used to mark germ cells. Our data indicated that DDX5 had lower level in PGCs at E9.5, but prominently expressed in gonocytes at E13.5 and E18.5 following sexual differentiation, as well as in postnatal germ cells (Figure 1E). Moreover, we observed that DDX5 expression was relatively weak in somatic cells from embryonic period. DDX5 was expressed in the nucleus. Together, these data indicate that DDX5 is not only abundant in the nucleus of germ cells after birth, but also in gonocytes in prenatal period, suggesting that DDX5 may regulate fate determination of germ cells at embryonic stages.

3.2 Germ cell-specific Ddx5 knockout leads to infertility

DDX5 is a multi-functional protein and is involved in several cellular processes. To further explore the role of Ddx5 in male germine development, Ddx5−/− mice were generated by breeding Ddx5 floxed mice (Ddx5flx/flox) with Mvh-Cre mice (Figure 2A). Ddx5+− and Ddx5−− mice were born followed Mendel's laws of inheritance and appeared healthy (Figure S1A). Western blotting showed that DDX5 protein was expressed in the testes of both wild-type and Ddx5−− adult mice but was faint in the testes of Ddx5−− adult mice.
**A**  
*Ddx5 mRNA*

Relative Expression Level

- P0: 0
- P5: 1
- P10: 2
- P15: 3
- P20: 4
- P25: 5
- P30: 6
- P90: 7

**B**  
*Mvh mRNA*

Relative Expression Level

- P0: 0
- P5: 1
- P10: 2
- P15: 3
- P20: 4
- P25: 5
- P30: 6
- P90: 7

**C**

| Gene | P0 | P5 | P10 | P15 | P20 | P25 | P30 | P90 |
|------|----|----|-----|-----|-----|-----|-----|-----|
| AKAP3 | 0  | 0  | 0   | 0   | 0.11| 1   | 0.98|     |
| MIWI  | 0  | 0  | 0   | 1   | 1.71| 1.58| 1.58| 1.91|
| SYCP3 | 0  | 0  | 0.19| 1   | 7.55| 6.63| 2.91| 1.99|
| DDX5  | 0  | 1  | 0.82| 1.16| 1.72| 2.81| 2.25| 1.99|

**D**

| Gene | SC | RS | ES |
|------|----|----|----|
| AKAP3 | 0  | 0.15| 1  |
| MIWI  | 1  | 1.49| 0.32|
| SYCP3 | 1  | 0   | 0  |
| DDX5  | 1  | 1.08| 0.02|

**E**

**Merge**

| Stage | DDX5 | Oct4-GFP | MVH | PLZF | TRA98 |
|-------|------|----------|-----|------|-------|
| E9.5  |      |          |     |      |       |
| E13.5 |      |          |     |      |       |
| E18.5 |      |          |     |      |       |
| P10   |      |          |     |      |       |
| P90   |      |          |     |      |       |

**Diagrams**

- **DDX5**
- **Oct4-GFP**
- **MVH**
- **PLZF**
- **TRA98**

**DAPI**
FIGURE 1  Expression analysis of Ddx5 during development of male germline. A and B, qRT-PCR to analyse the expression of Ddx5 and germ cell marker Mvh in the testes of mice at different stages (P0 to P90). Expression levels were normalized against geometric mean of Gapdh and Actin. Error bars correspond to means ± SD (*P < .05, **P < .01, ***P < .001). C, Western blotting to analyse DDX5 protein in the testes of mice at different stages (P0 to P90). AKAP3 was used as elongating spermatid marker. MIWI was used as spermatocyte and round spermatid marker. SYCP3 was used as spermatocytes marker. β-ACTIN was used as a loading control. D, Western blotting to analyse protein level of AKAP3, MIWI, SYCP3 and DDX5 in the fractionated testicular spermatocytes (SC), round spermatids (RS) and elongating spermatids (ES). β-ACTIN was used as a loading control. E, Immunofluorescent staining of DDX5 expression in germ cells from wild-type mice at E9.5 to P90. Germ cells were labelled with Oct4-GFP (green) in E9.5 and E13.5 gonad sections. Spermatogonia were labelled with anti-PLZF antibody in testis section at P10. All germ cells except elongating spermatids were labelled with anti-TRA98 antibody in testis section at P90. DNA was stained with DAPI (blue). Scale bars represent 20 μm.

FIGURE 2  Ddx5 is essential for spermatogenesis. A, Schematic diagram of Ddx5 conditional knockout strategy. Left panel, the Ddx5 allele is flanked by two loxP sites. The blue squares stand for Ddx5 exon, and the red triangles represent loxP site. Right panel, reproductive strategy for Ddx5+/− mice. B, Western blotting to detect protein level of DDX5, MIWI, MVH and SYCP3 in the testes of wild-type (WT), Ddx5+/− and Ddx5−/− adult mice. β-ACTIN was used as a loading control. C, Morphological analysis of testes in WT, Ddx5+/− and Ddx5−/− adult mice. Scale bar represents 5 mm. D, Assessment of testis to body weight ratio (mg/mg) from WT, Ddx5+/− and Ddx5−/− adult male mice. Error bars correspond to means ± SD. P = .4428 between WT and Ddx5+/−, P = 3.644e−05 between Ddx5+/− and Ddx5−/− and P = 7.396e−07 between WT and Ddx5−/− (***P < .001, n = 6). E, H&E staining of testis sections from WT, Ddx5+/− and Ddx5−/− adult mice. The gaps between the seminiferous tubules due to the processing of testis section preparation. Scale bars represent 100 μm and 10 μm, respectively. SC, spermatocytes, ES, elongating spermatids, RS, round spermatids, SSC, spermatogonial stem cells, Sertoli, Sertoli cells. F, H&E staining of cauda epididymis sections from WT, Ddx5+/− and Ddx5−/− adult male mice. Scale bars represent 100 μm and 50 μm, respectively. G, Quantification of sperms released from cauda epididymides of WT, Ddx5+/− and Ddx5−/− adult males. Error bars correspond to means ± SD. P = .6646 between WT and Ddx5+/−, P = 1.027e−05 between Ddx5+/− and Ddx5−/−, and P = 1.467e−10 between WT and Ddx5−/− (***P < .001, n = 6)
(Figure 2B and Figure S1C). And strikingly, no pups were born when Ddx5−/− male mice mated with wild-type female mice, even copulatory plugs were routinely observed (Table 1). Together, these data reveal that germ cell-specific knockout of Ddx5 leads to male infertility.

### 3.3 | Ddx5 deletion results in the depletion of germ cells in adult male mice

To assess the reason of infertility in Ddx5-deficient male mice, we observed that the testes of Ddx5−/− mice were significantly smaller and lighter than those of wild-type and Ddx5+/− mice (Figure 2C,D). Haematoxylin and eosin (H&E) staining showed that although the architecture of seminiferous tubules largely preserved, all tubules were atrophy and empty in the testes of Ddx5−/− mice compared with multiple layers spermatogenic cells in the testes of wild-type and Ddx5+/− mice (Figure 2E). In addition, Ddx5−/− mice resulted in loss of mature sperms in the cauda epididymides (Figure 2F,G). Consistently, germ cell-specific markers were not detectable in Ddx5−/− mice (Figure 2B and Figure S1C). Meanwhile, flow cytometry analysis identified stage-specific subpopulations of spermatogenic cells in wild-type, but not in Ddx5−/− mice (Figure S1B). The above data demonstrate that Ddx5−/− adult male mice have no capacity to produce germ cells.

### 3.4 | Ddx5 deletion results in a Sertoli cell-only phenotype in adult male mice

Although seminiferous tubules were empty in Ddx5−/− adult mice, there was still one layer of cells in peripheral regions of seminiferous tubules. It has been reported that Sertoli cells and SSCs are located in the basement membrane. To examine the identity of the cells remained in seminiferous tubules, we performed immunofluorescent staining and observed that Ddx5−/− mice resulted in loss of TRA98-positive germ cells compared with wild-type and Ddx5+/− mice, but only SOX9-positive Sertoli cells left (Figure 3A,B). The phenotype that Ddx5−/− adult male mice were completely devoid of germ cells is consistent with Sertoli cell-only syndrome associated with human infertility. SSCs could not maintain in Ddx5−/− adult mice, we wondered whether Ddx5 affects the source of SSCs. We compared the testes in wild-type and Ddx5−/− mice at P6. Immunofluorescent staining with TRA98 indicated that SSCs were completely depleted in Ddx5−/− seminiferous tubules (Figure 3C). Therefore, these results indicate that Ddx5 regulates male germline development at the early stage, even before spermatogonia pool is formed at P6.

### 3.5 | Germ cells are gradually lost in testes of Ddx5−/− neonates

Mvh-Cre transgenic mice express recombinase faintly at E15 and strongly at E18. To explore the effect of Ddx5 on germ cells during the embryonic stage, we collected testes at E18.5 and found that Ddx5 knockout has no effect on the number of gonocytes (Figure S2A,B). Therefore, we focused on the effect of Ddx5 loss on postnatal days. We observed that Oct4-GFP-positive germ cells were reduced in the testes of Ddx5−/− mice at P0 and P2, and were totally absent at P6. In contrast, no change was observed in oocytes between wild-type and Ddx5−/− mice (Figure 4A). Additionally, immunofluorescent staining revealed that Ddx5−/− mice had significantly reduced numbers of gonocytes in seminiferous tubules at P0 (Figure 4B), and about 42% gonocytes were diminished in the testes of Ddx5−/− mice (Figure 4C). We also analysed the percentage of Oct4-GFP-positive cells using flow cytometry at P2. Noticeably, Ddx5−/− mice had only 0.11% gonocytes in all testicular cells while wild-type mice had 1.31% (Figure 4D). Taken together, these results demonstrate that gonocytes are gradually reduced and then completely depleted from E18.5 to P6 upon Ddx5 ablation, suggesting that Ddx5 is indispensable for the survival of gonocytes in postnatal testes.

### 3.6 | Single-cell RNA sequencing analysis reveals the effects of Ddx5 knockout on gene expression in gonocytes

To investigate the regulatory mechanism of Ddx5 in gonocytes, scRNA-seq analysis was performed on dissociated testicular cells, which were isolated from whole testes of wild-type and Ddx5−/− mice at P2 (Figure 5A). We obtained 6328 and 4950 single-cell transcriptomes in the testes of wild-type and Ddx5−/− mice after quality control, respectively (Figure S3A). Dimensionality reduction and visualization using uniform manifold approximation and projection (UMAP) allowed us to divide the cells into eight distinct clusters designated as clusters 1 to 8 that can be annotated into germ cells, peritubular macrophages, peritubular myoid cells, Leydig cells and

---

**TABLE 1** The fertility of Ddx5 conditional knockout male mice

| Genotype        | Male | Female | NO. of male mice | NO. of plugged female mice | NO. of litters | NO. of pups per litter |
|-----------------|------|--------|------------------|---------------------------|----------------|------------------------|
| WT              | WT   | 7      | 24               | 184                       | 7.67           |
| Ddx5+/−         | WT   | 7      | 24               | 175                       | 7.29           |
| Ddx5+/−         | WT   | 7      | 24               | 0                         | 0              |

Note: 1. Number. 2. Wild-type. 3. Heterozygotes Ddx5+/−. 4. Homozygotes Ddx5−/−.
Sertoli cells according to known marker genes (Figure S5B,C; Figure S3B,C; Table S3). The relative proportion of each cluster weighed by the percentage of total cells in both genotypes showed that the ratio of germ cells in the testes was dropped dramatically from 1.86% to 0.24% in Ddx5<sup>-/-</sup> mice compared with wild type, while the other cell clusters remained unchanged (Figure S3D).
To further elucidate the effect of Ddx5 loss on cellular responses in the testes, germ cells (gonocytes) were selected for further analysis. 54 germ cells (47 from wild type and 7 from Ddx5<sup>−/−</sup>) were subjected to principal component analysis (PCA) based on the highly variable genes (n = 3817). We found a segregated distribution for wild-type and Ddx5<sup>−/−</sup> cells along the PC1 (Figure 5D).

The differentially expressed gene analysis identified 848 genes including 232 up-regulated genes and 616 down-regulated genes in the gonocytes of Ddx5<sup>−/−</sup> mice (Figure 5E), confirming a profound change in gene expression profiles. Gene Ontology (GO) analysis revealed that the up-regulated genes were mainly related to the formation of free 40S subunits, ribonucleoprotein
complex assembly and mRNA metabolic process (Figure S3E), while the down-regulated genes were significantly associated with mitotic cell cycle, cell cycle phase transition and DNA repair (Figure 5F,G). Altogether, scRNA-seq analysis reveals that Ddx5 knockout obviously disturbs the expression of genes in gonocytes.

3.7 Ddx5 maintains survival of gonocytes through regulating the expression level of genes related to GDNF pathway

After Ddx5 knockout, the most majority of differentially expressed genes (DEGs) were down-regulated, and many of them were critical
**FIGURE 6**  
*Ddx5* maintains gonocytes and regulates the transcription of genes in GDNF pathway.  
(A) Schematic diagram showing GDNF-GFRα1-RET signaling pathway in the gonocytes.  
(B) Single-cell qRT-PCR to analyse the expression of *Ddx5* and GDNF-GFRα1-RET signaling pathway-related genes in *Oct4*-GFP-positive gonocytes sorted by FACS in WT and *Ddx5*−/− testicular cells at P2. Expression levels were normalized against geometric mean of *Gapdh* and *Actin*. Error bars correspond to means ± SD (***, *P* < .001, n = 3).  
(C and D) Immunofluorescent staining of GFRα1 and Ki67 in WT and *Ddx5*−/− testes at P2, respectively. Quantification of immunofluorescent signals of GFRα1 and Ki67 per unit area in the testis sections of WT and *Ddx5*−/− male mice at P2, respectively, was calculated in the right panels. The percentage indicates the proportion of GFRα1 or Ki67-positive gonocytes to all gonocytes in the testis sections. GFRα1−: GFRα1-negative gonocytes, GFRα1+: GFRα1-positive gonocytes, Ki67−: Ki67-negative gonocytes, Ki67+: Ki67-positive gonocytes. Error bars correspond to means ± SD (n = 3). Gonocytes were labelled with *Oct4*-GFP (green). DNA was stained with DAPI. Scale bars represent 20 μm.
for male germ cell development, including components in GDNF signaling pathway (Figure 5G). GDNF is a growth factor secreted by testicular somatic cells, which is necessary for spermatogonia proliferation and survival. GDNF forms a complex with GFRα1, a cell surface receptor of the gonocytes, and subsequently binds to the transmembrane RET tyrosine kinase and activates intracellular signaling pathways (Figure 6A). Our scRNA-seq data showed that the GDNF pathway-related genes were down-regulated in Ddx5−/− male mice (Figure 5G). To confirm the dysregulation of GDNF pathway following Ddx5 knockout, we performed single-cell qRT-PCR analysis on Oct4-GFP-positive male germ cells, which were isolated from the testes of both wild-type and Ddx5−/− males at P2. Our data showed that the mRNA levels of Ddx5, Gfra1, Ret, Gdnf, Pou3f1, Fyn, Id4 and Etv5 were dramatically decreased in Ddx5-deficient gonocytes (Figure 6B), which is consistent with the scRNA-seq results. Moreover, immunofluorescent staining of cross-sections showed that GFRα1 protein level was significantly reduced on the surface of gonocytes in Ddx5−/− testes compared with that in wild type (Figure 6C). Meanwhile, we barely observed proliferating gonocytes in the newborn Ddx5−/− mice by immunofluorescent staining with anti-Ki67 antibody (Figure 6D). We also examined possible gonocyte apoptosis through TUNEL staining and found that apoptotic cells did not increase in Ddx5−/− neonates (Figure 5C). In summary, these data suggest that Ddx5 knockout disrupts the GDNF signaling pathway and might further lead to phenotypic defects in proliferation and survival of gonocytes.

4 | DISCUSSION

RNA-binding protein DDX5 is widely known to participate in various aspects of RNA metabolism. In this study, we report that Ddx5 is required for male germ cell development and Ddx5-deficient male mice are infertile due to loss of gonocytes in neonates. The phenotype of Ddx5−/− mice is similar to the Sertoli cell–only syndrome (SCO) in humans, which shows the complete absence of germ cells in human testicular tissues. While the potential mechanism of SCO is still unclear, DDX5 loss might contribute to SCO in humans.

During the process of our study, another independent laboratory reported UBC-CreERT2 mediated Ddx5 knockout affects the maintenance of spermatogonia. Ddx5 knockout in spermatogonia at 8 to 12 weeks led to completely devoid of germ cells in seminiferous tubules. Here, we generated Ddx5 conditional knockout mice by using Mvh-Cre to delete Ddx5 from embryonic period and found that the infertility of adult male mice after Ddx5 loss was due to low survival of gonocytes. Based on theirs and our study, both of us acquired the similar Sertoli cell–only phenotype and male infertility in adult mice. However, compared with the discoveries from Legrand et al., we mainly focused on the roles of Ddx5 in gonocytes at the early stage from E18.5 to P6. Indeed, our data clearly indicated that Ddx5 was important for the survival and transition of gonocytes. However, it remains unknown how Ddx5 regulates gene expression of gonocytes. DDX5 is widely known to be involved in several steps of RNA metabolism and ribosome biogenesis, in which many genes were dysregulated in Ddx5−/− gonocytes. Further, the significantly enriched pathways of down-regulated genes were cell cycle-related pathways, which were consistent with the findings in spermatogonia. Moreover, many genes involved in male germ cell development were significantly down-regulated in gonocytes of Ddx5−/− mice, including almost all of the genes related to GDNF-GFRα1-RET signaling pathway (eg, Gfra1, Ret, Etv5 and Id4) and spermatogenesis-related genes (eg, Zbb16, Sall4, Gtsf1 and Dmr1). Among them, ZBBT16 and SALL4 are two important transcription factors for self-renewal and differentiation of spermatogonia. In addition, both Gtsf1 and Dmr1 are vital for spermatogenesis beyond the early meiotic phase. Therefore, additional mechanisms for Ddx5 in regulating survival of neonatal mouse gonocytes could be pursued by investigating how Ddx5 regulates the expression of Zbb16, Sall4, Gtsf1 and Dmr1.

In conclusion, the findings presented here demonstrate that Ddx5 is required and essential for male fertility. Furthermore, Ddx5 is indispensable for survival and transition of gonocytes in male neonates at least through regulating the genes that associated with cell cycle and GDNF pathway.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science and Technology of the People’s Republic of China (2016YFA0100300), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA16010502, XDA16020404), the Ministry of Science and Technology of the People’s Republic of China (2016YFA0100400), National Natural Science Foundation of China (31925009, 81902885, 32000424, 31871456), Key Research & Development Program of Guangzhou Regenerative Medicine and Health Guangdong Laboratory (2018GZR110104007), Science and Technology Planning Project of Guangdong Province, China (2019B020234004, 2020B1212060052), Science and Technology Program of Guangzhou, China (201906010096), and Guangdong Basic and Applied Basic Research Foundation (2019A1515110028, 2019B151502054). The authors also gratefully thank the support from the Guangzhou Branch of the Supercomputing Center of the Chinese Academy of Sciences.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

HY and QX initiated the study and designed the experiments. QX conducted most of the experiments. GC performed the bioinformatics analysis. YF provided the Ddx5 floxed mice. XW, GH, LW, XL, LY, QC, KX, WG, MG and YL provided necessary assistance. JW and WL provided Mvh-Cre transgenic mice. JC and HQ contributed to the work. HY, QX and GC wrote the manuscript. HY and GP conceived and supervised the entire study. HY approved the final version.
DATA AVAILABILITY STATEMENT
RNA-seq data generated in this study are deposited in the NCBI Gene Expression Omnibus under accession number GSE158285.

ORCID
Guizhong Cui https://orcid.org/0000-0001-6394-2773
Guangrun Peng https://orcid.org/0000-0002-8586-0637
Hongjie Yao https://orcid.org/0000-0003-2144-0147

REFERENCES
1. Saitou M, Barton SC, Surani MA. A molecular programme for the specification of germ cell fate in mice. Nature. 2002;418:293-300.
2. Saitou M. Specification of the germ cell lineage in mice. Front Biodiv (Landmark Ed). 2009;14:1068-1087.
3. Guenesdogan U, Magnusdottr E, Surani MA. Primordial germ cell specification: a context-dependent cellular differentiation event [corrected]. Philos Trans R Soc Lond B Biol Sci. 2014;369:20130543.
4. Anderson R, Copeland TK, Scholer H, et al. The onset of germ cell migration in the mouse embryo. Mech Dev. 2000;91:61-68.
5. Ginsburg M, Snow MH, McLaren A. Primordial germ cells in the mouse embryo during gastrulation. Development. 1990;110:521-528.
6. McLaren A. Primordial germ cells in the mouse. Dev Biol. 2003;262:1-15.
7. Harikae K, Miura K, Kanai Y. Early gonadogenesis in mammals: significance of long and narrow gonadal structure. Dev Dyn. 2013;242:330-338.
8. Barton LJ, LeBlanc MG, Lehmann R. Finding their way: themes in particular germ and somatic cell development during the perinatal period. Int J Androl. 2006;26:339-347.
9. Yamanaka S, Nishiara H, Toh H, et al. Broad Heterochromatic Domains Open in Gonocyte Development Prior to De Novo DNA Methylation. Dev Cell. 2019;51:21-34.e5.
10. Kluin PM, de Rooy DG. A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. Int J Androl. 1981;4:475-493.
11. Yoshida S, Suzuki M, Nakagawa T, et al. The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development. 2006;133:1495-1505.
12. Tan K, Song HW, Wilkinson MF. Single-cell RNAseq analysis of testicular germ and somatic cell development during the perinatal period. Development. 2020;147(3):dev183251.
13. McCarrey JR. Toward a more precise and informative nomenclature describing fetal and neonatal male germ cells in rodents. Biol Reprod. 2013;89:47.
14. Gnesi L, Fabbrì A, Spera G. Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. Endocr Rev. 1997;18:541-609.
15. Basciani S, De Luca G, Dolci S, et al. Platelet-derived growth factor receptor beta-subtype regulates proliferation and migration of gonocytes. Endocrinology. 2008;149:6226-6235.
16. Neuhus N, Yoon J, Terwort N, et al. Single-cell gene expression analysis reveals diversity among human spermatogonia. Mol Hum Reprod. 2017;23:79-90.
17. O’Bryan MK, Clark BJ, McLaughlin EA, et al. RBMS is a male germ cell splicing factor and is required for spermatid differentiation and male fertility. PLoS Genet. 2013;9:e1003628.
18. Zhang H, Wang G, Liu L, et al. KH-type splicing regulatory protein A is a new component of chromatoid body. Reproduction. 2017;154:723-733.
19. Arun G, Akhade VS, Donakonda S, et al. mrhl RNA, a long noncoding RNA, negatively regulates Wnt signaling through its protein partner Ddx5/p68 in mouse spermatogonial cells. Mol Cell Biol. 2012;32:3140-3152.
20. Legrand JMD, Chan AL, La HM, et al. DDX5 plays essential transcriptional and post-transcriptional roles in the maintenance and function of spermatogonia. Nat Commun. 2019;10:2278.
21. Nicol SM, Bray SE, Black HD, et al. The RNA helicase p68 (DDX5) is selectively required for the induction of p53-dependent p21 expression and cell-cycle arrest after DNA damage. Oncogene. 2013;32:3461-3469.
22. Gallardo T, Shirley L, John GB, et al. Generation of a germ cell-specific mouse transgenic Cre line. Vasa-Cre. Genesis. 2007;45:413-417.
23. Bustin SA. Why the need for qPCR publication guidelines?–The case for MIQE. Methods. 2010;50:217-226.
24. Chen J, Suo S, Tam PP, et al. Spatial transcriptomic analysis of cryosectioned tissue samples with Geo-seq. Nat Protoc. 2017;12:566-580.
25. Bellve AR, Cavacchia JC, Millette CF, et al. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol. 1977;74:68-85.
26. Fuller-Pace FV, Moore HC. RNA helicases p68 and p72: multifunctional proteins with important implications for cancer development. Future Oncol. 2011;7:239-251.
27. Xing Z, Ma WK, Tran EJ. The DDX5/Dbp2 subfamily of DEAD-box RNA helicases. Wiley Interdiscip Rev RNA. 2019;10:e1519.
28. Chen SR, Liu YX. Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling. Reproduction. 2015;149:R159-R167.
29. Becht E, McInnes L, Healy J, et al. Dimensionality reduction for visualizing single-cell data using UMAP. Nat Biotechnol. 2019;37(1):38-44.
30. Chen LY, Willis WD, Eddy EM. Targeting the Gdnf Gene in peritubular myoid cells disrupts undifferentiated spermatogonial stem cell development. Proc Natl Acad Sci USA. 2016;113:1829-1834.
31. Naughton CK, Jain S, Strickland AM, et al. Giall cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod. 2006;74:314-321.
32. Pui HP, Saga Y. Gonocytes-to-spermatogonia transition initiates prior to birth in murine testes and it requires FGF signaling. Mech Dev. 2017;144:125-139.
33. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci. 2002;3:383-394.
34. Ramphul K, Mejias SG. Sertoli-Cell-Only Syndrome. Treasure Island, FL: StatPearls; 2020.
35. Saporita AJ, Chang HC, Winkeler CL, et al. RNA helicase DDX5 is a p53-independent target of ARF that participates in ribosome biogenesis. Cancer Res. 2011;71:6708-6717.
36. Jalal C, Uhlmann-Schiffer H, Stahl H. Redundant role of DEAD box proteins p68 (Ddx5) and p72/p82 (Ddx17) in ribosome biogenesis and cell proliferation. Nucleic Acids Res. 2007;35:3590-3601.
37. Costoya JA, Hobs RM, Barna M, et al. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet. 2004;36:653-659.
38. Chan AL, La HM, Legrand JMD, et al. Germline Stem Cell Activity Is Sustained by SALL4-Dependent Silencing of Distinct Tumor Suppressor Genes. Stem Cell Reports. 2017;9:956-971.
39. Hobs RM, Fagonee S, Papa A, et al. Functional antagonism between Sall4 and Plzf defines germline progenitors. Cell Stem Cell. 2012;10:284-298.
40. Hobs RM, Seandel M, Falcatori I, et al. Plzf regulates germline progenitor self-renewal by opposing mTORC1. Cell. 2010;142:468-479.
41. Yoshimura T, Toyoda S, Kuramochi-Miyagawa S, et al. Gtsf1/Cue110, a gene encoding a protein with two copies of a CHHC
Zn-finger motif, is involved in spermatogenesis and retrotransposon suppression in murine testes. *Dev Biol*. 2009;335:216-227.

45. Raymond CS, Murphy MW, O'Sullivan MG, et al. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev*. 2000;14:2587-2595.

46. Krentz AD, Murphy MW, Kim S, et al. The DM domain protein DMRT1 is a dose-sensitive regulator of fetal germ cell proliferation and pluripotency. *Proc Natl Acad Sci USA*. 2009;106:22323-22328.

47. Fahrioglu U, Murphy MW, Zarkower D, et al. mRNA expression analysis and the molecular basis of neonatal testis defects in Dmrt1 mutant mice. *Sex Dev*. 2007;1:42-58.

**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Xia Q, Cui G, Fan Y, et al. RNA helicase DDX5 acts as a critical regulator for survival of neonatal mouse gonocytes. *Cell Prolif*. 2021;54:e13000. [https://doi.org/10.1111/cpr.13000](https://doi.org/10.1111/cpr.13000)