Am J Transl Res 2024;16(5):1834-1844
www.ajtr.org /ISSN:1943-8141/AJTR0157153

Original Article

E3 ligase FBXO22 is not significant for spermatogenesis and male fertility in mice

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Received April 11, 2024; Accepted May 15, 2024; Epub May 15, 2024; Published May 30, 2024

Abstract: Background: F-box-only protein 22 (FBXO22), an important substrate receptor of the SKP1-Cullin-F-box (SCF) ubiquitin ligases, has been reported to be involved in many biological processes, including tumorigenesis, neurological disorders, cellular senescence, and DNA damage. However, the specific role of FBXO22 during spermatogenesis is poorly understood. Methods: We produced *Fbxo22* conditional knockout (cKO) and global knockout (KO) mice and assessed their sperm masurements using a computer-assisted sperm analysis (CASA) system. Additionally, we conducted histologic staining and immunostaining to examine the impact of *Fbxo22* loss on spermatogenesis. Results: Our results revealed that there were no notable differences in semen quality, fertility test results, or histologic findings in *Fbxo22*-KO and *Fbxo22*-cKO mice compared to the control group. Conclusions: Our study demonstrated that *Fbxo22* is not significant for spermatogenesis or male fertility in mice. These findings will help researchers avoid redundant efforts and serve as a foundational resource for genetic studies on human fertility.

Keywords: *Fbxo22*, spermatogenesis, knockout, male fertility

Introduction

Spermatogenesis is a highly orchestrated process involving spermatogonial proliferation, spermatocyte meiosis, and spermatid differentiation to produce mature spermatozoa [1]. Ubiquitination, a significant posttranslational modification in eukaryotes regulating protein stability and activity, governs cell remodeling and protein turnover throughout various phases of spermatogenesis, spanning from the gonocyte to the mature spermatozoa stage [2-5]. Protein ubiquitination operates through a series of enzymatic reactions involving the ubiquitin-activating E1 enzyme, the ubiquitin-conjugating E2 enzyme, and the ubiquitin-protein E3 ligase. Importantly, the E3 ligase specifies the particular substrates marked for ubiquitination and eventual degradation [6, 7].

In mammals, the most extensively investigated E3 ligase complex is the S-phase kinase-associated protein 1 (SKP1)-Cullin 1 (CUL1)-F-box protein (SCF) complex, which is a member of the Cullin-RING ligases family. This complex CUL1, SKP1, ring box protein 1 (RBX1), and F-box proteins [8, 9]. F-box-only protein 22 (FBXO22), a member of the F-box family, is implicated in cell cycle regulation, DNA damage response, signal transduction, and apoptosis [10, 11]. Aberrant FBXO22 expression is intricately linked to tumorigenesis. For example, FBXO22 has been reported to promote the malignant progression of glioblastoma by tar-

https://doi.org/10.62347/STDA4237

geting von Hippel-Lindau disease tumor suppressor (VHL) for degradation [12]. Moreover, studies have disclosed that FBXO22 can promote cervical cancer progression by mediating the ubiquitination and proteasomal degradation of $p57^{Kip2}$ [13]. In contrast to the role of its oncogene in cancer progression, FBXO2 also exerts tumor-suppressive effects. Previous studies have shown that FBXO22 mediates the ubiquitination-dependent degradation of cyclin-G-associated kinase (GAK), leading to the inhibition of proliferation and metastasis in cervical cancer cells [14]. Furthermore, FBXO22 suppresses metastasis in triple-negative breast cancer by modulating lysine-specific demethylase 5A (KDM5A) through ubiquitin modification, thereby regulating H3K4me3 demethylation [15].

The present study was designed to systematically explore a possible role of FBXO22 during mouse spermatogenesis. To achieve this goal, we generated two *Fbxo22* conditional knockout (cKO) mouse strains and one global knockout (KO) strain and found that *Fbxo22* was not significant for spermatogenesis and male fertility in mice.

Materials and methods

Bioinformatic analysis

Two scRNA-seq datasets were obtained from the Gene Expression Omnibus database (GSM5563668 and GSE149512). These datasets contain information on normal adult testis samples from mice and humans, respectively. Data analysis was conducted using the "Seurat" packages. Scaled data were integrated using the "Harmony" function. Cell types were characterized by marker genes. Gene expression and distribution were displayed using "Dotplot" and "Featureplot".

Animals

Fbxo22$^{flox/+}$ mice were purchased from Cyagen Biosciences Inc. (Suzhou, China), and *Amh/Stra8-Cre* transgenic mice were obtained from the State Key Laboratory of Reproductive Medicine and Offspring Heath of Nanjing Medical University. These mutant mouse strains had a mixed background of ICR and C57BL/6J. The mice were raised under specific pathogen-free conditions at the Animal Center of Nanjing Medical University, and the experiments involving these mice were approved by the Animal Ethics and Welfare Committee of Nanjing Medical University (Approval No. 2004020).

Polymerase chain reaction (PCR) genotyping

Genotyping of *Fbxo22*-wild-type (WT), *Fbxo22*-gKO, *Fbxo22*-sKO, and *Fbxo22*-KO mice was performed by PCR and agarose gel electrophoresis of DNA. The primers used for genotyping were as follows: F1: 5'-CTCAGAGTTGAGACTACACAAGCA-3'; R1: 5'-GTTCAGTATTTCAGACCTGCTCAC-3'; R2: 5'-GTCCTCCAAAGACACAATTCACTG-3'.

Fertility testing

To evaluate fertility, the control group and *Fbxo22* mutant males were paired with female WT mice (all aged 8 weeks) at a ratio of 1:2 for 3 months, and litter sizes per litter were recorded.

Histology

We collected testes from 8- to 10-week-old *Fbxo22*-WT, *Fbxo22*-gKO, *Fbxo22*-sKO, and *Fbxo22*-KO mice, and the tissues were fixed in modified Davidson's fluid for 48 h. Subsequently, the tissues were dehydrated in a series of ethanol solutions, embedded in paraffin, and sectioned at 5 μm thickness. The sections were then rehydrated and stained with hematoxylin and eosin (H&E) as previously described [16, 17], images were captured using an optical microscope, and histological analysis was performed.

Computer-assisted sperm analysis (CASA)

Sperm from 8- to 10-week-old *Fbxo22*-WT, *Fbxo22*-gKO, *Fbxo22*-sKO, and *Fbxo22*-KO mice were collected from the cauda epididymis, suspended in human tubal fluid culture medium (InVitroCare, Inc., Frederick, MD, USA), incubated at 37°C for 5 min, and then assessed for semen quality using a Ceros™ II sperm analysis system (Hamilton Thorne, Beverly, MA, USA).

Immunofluorescence

Immunofluorescence was performed according to our previously reported protocol with minor

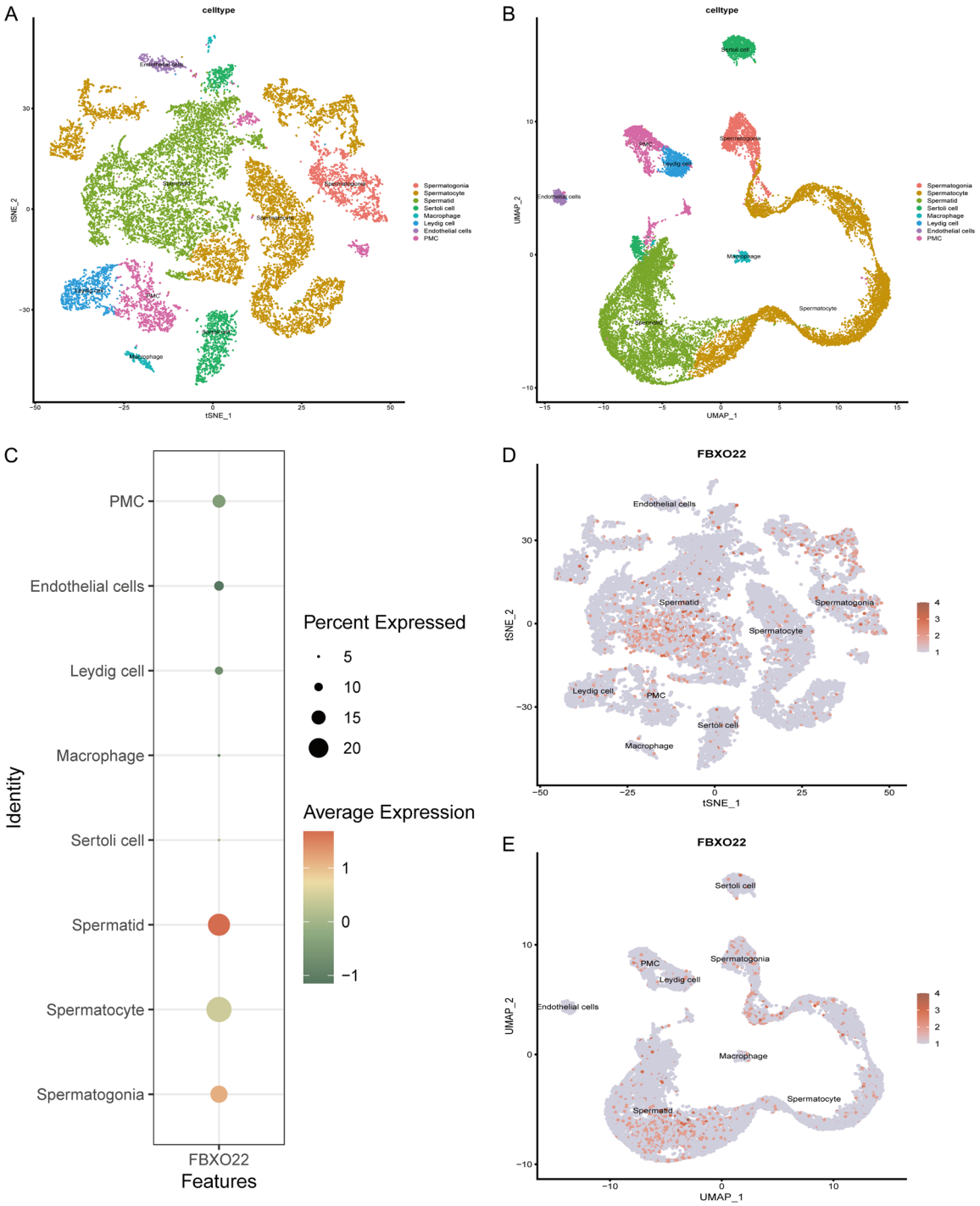

Figure 1. Expression and distribution of *FBXO22* in normal human adult testis samples. (A, B) tSNE (A) and UMAP (B) results display the cell subpopulations in the testis samples. (C) Dotplot illustrating *FBXO22* expression in various cell types from the testis samples. (D, E) tSNE (D) and UMAP (E) featureplots show the distribution of *FBXO22* in various cells from the testis samples.

modifications [18-20]. Briefly, sections were subjected to antigen retrieval in a 10 mM citrate buffer (pH 6.0). After blocking with 1% (w/v) bovine serum albumin for 2 h, the sections were incubated with the following primary antibodies: anti-3β-hydroxysteroid dehydrogenase (3β-HSD) (1/500, Santa), anti-synaptonemal complex protein 3 (SCP3) (1/200, Abcam),

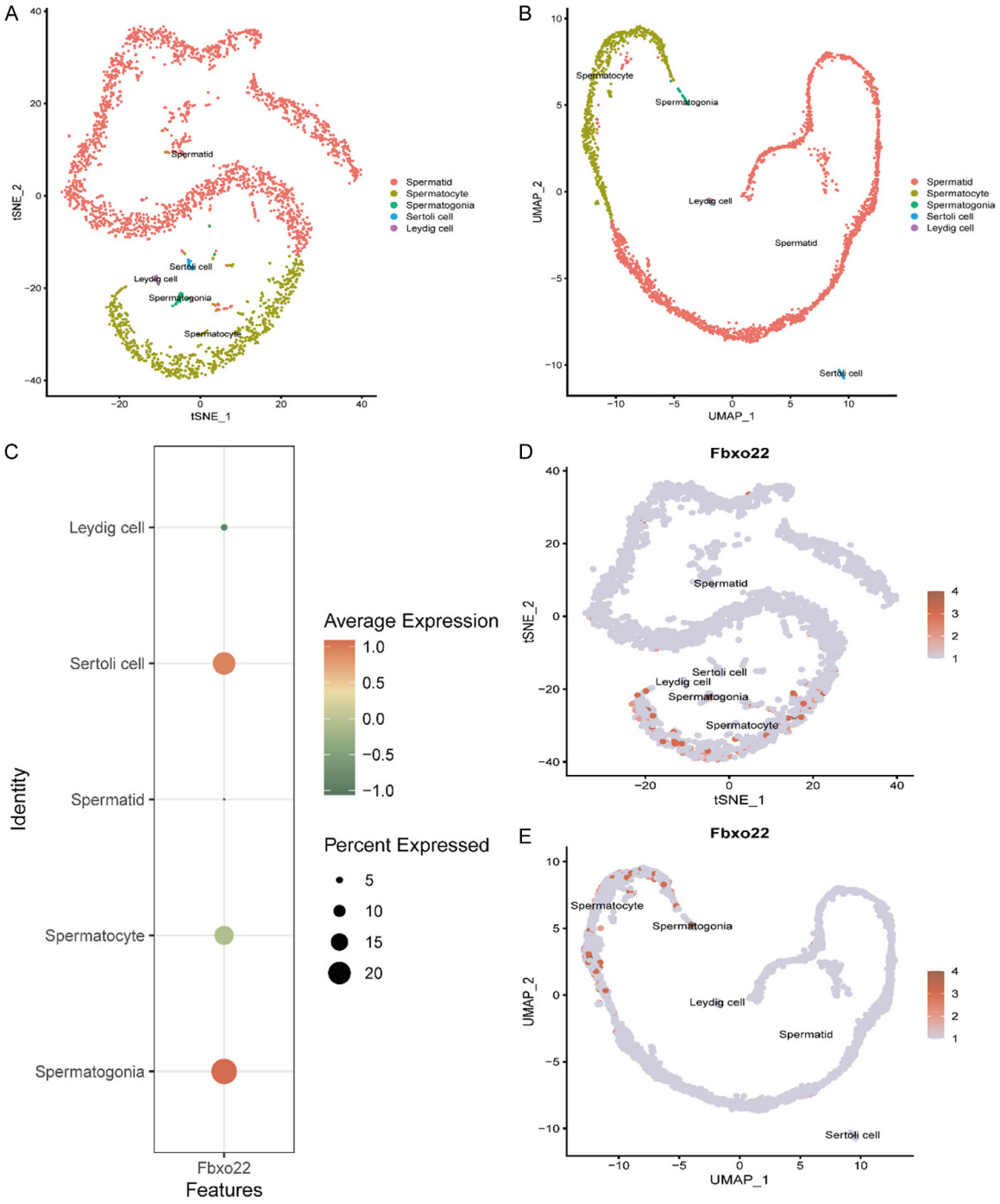

Figure 2. Expression and distribution of *Fbxo22* in normal adult mouse testis samples. (A, B) tSNE (A) and UMAP (B) results display the cell subpopulations of the testis samples. (C) Dotplot illustrating the expression of *Fbxo22* in various cells from the testis samples. (D, E) tSNE (D) and UMAP (E) featureplots showing the distribution of *Fbxo22* in various cell types from the testis samples.

anti-Lin28 (1/200, Abcam), anti-SRY-box transcription factor 9 (SOX9) (1/200, Millipore) and peptide nucleic acid (PNA) (1/500, Vector) at 4°C for 12 to 16 hours. Subsequently, sections were gently washed three times with phosphate-buffered saline at room temperature and then incubated at 37°C for 1 h with Alexa-Fluor secondary antibodies (Thermo Scientific, Waltham, USA). Finally, the sections were stained with 4',6-diamidino-2-phenylindole

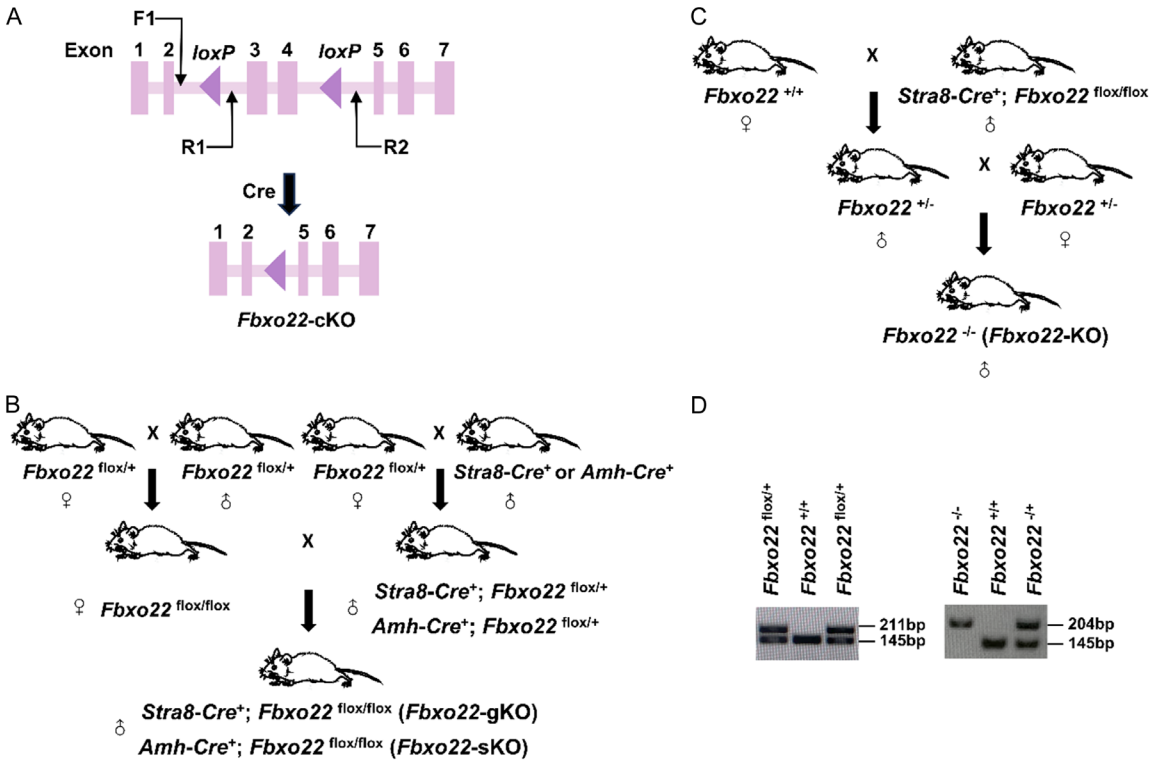

Figure 3. Generation of *Fbxo22*-cKO/KO strains. (A) Schematic diagram of the conditional knockout of *Fbxo22*. (B, C) Breeding strategies for producing *Fbxo22* conditional knockout mice (B) and global knockout mice (C). (D) Validation of mouse genotypes by PCR.

(DAPI), images were captured using an LSM800 confocal microscope (Zeiss, Oberkochen, Germany), and fluorescence analysis was performed.

Statistical analysis

Statistical analysis was performed using Prism 8.0 (GraphPad, La Jolla, CA, USA). The significance of differences was calculated using the unpaired Student's t-test or analysis of variance. $P < 0.05$ was considered significant.

Results

FBXO22/Fbxo22 expression patterns in human and mouse testes

FBXO22, which features the hallmark F-box domain, is a conserved protein across species (Supplementary Figure 1). Due to the lack of effective antibodies targeting FBXO22, we relied on bioinformatic predictions to ascertain the expression and distribution of FBXO22 within the testes. Using publicly available single-cell RNA sequencing (scRNA-seq) data from human and mouse testes, our analysis revealed distinct patterns of *FBXO22/Fbxo22* expression. In human testes, *FBXO22* was predominantly expressed in spermatogonia, spermatocytes, and spermatids (**Figure 1**). Conversely, in mouse testes, *Fbxo22* exhibited significant expression in spermatogonia, spermatocytes, and Sertoli cells, with negligible expression observed in spermatids (**Figure 2**).

Generation of Fbxo22-cKO/KO mice

As *Fbxo22* demonstrated expression in both germ cells and Sertoli cells in mouse testes, we proceeded to generate germ cell- and Sertoli cell-specific KO mouse models for *Fbxo22*. As shown in **Figure 3A**, exons 3-4 of the *Fbxo22* gene were flanked with *loxP* sites by clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR/Cas9)-based genomic editing, which led to the deletion of exons 3-4 under the control of Cre recombinase. Then, by crossing *Fbxo22*-floxed mice with *Stra8-Cre* and *Amh-Cre* transgenic mice, respectively, we obtained germ cell- and Sertoli cell-specific *Fbxo22*-knockout (referred

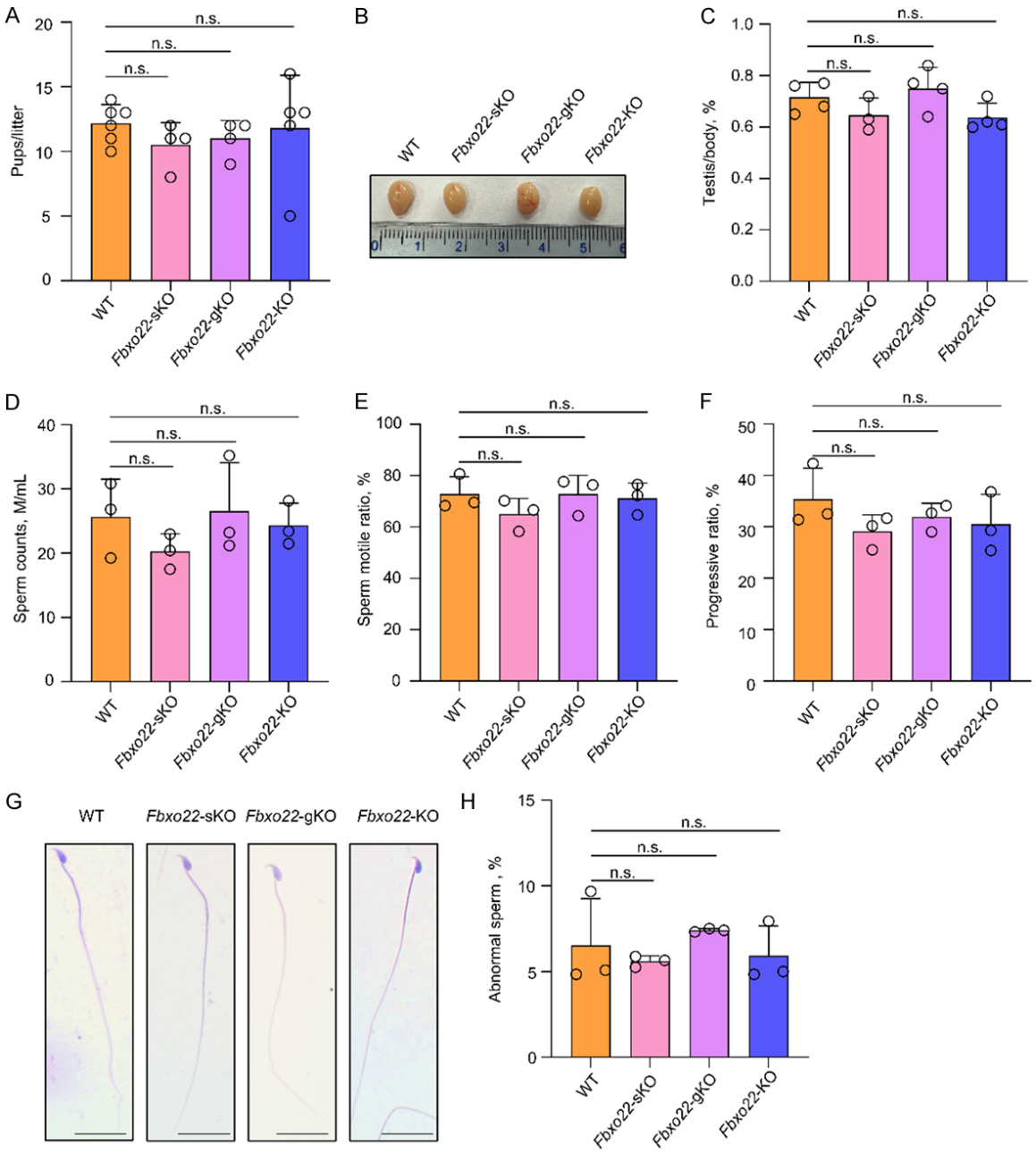

Figure 4. Normal fertility in *Fbxo22*-sKO, *Fbxo22*-gKO, and *Fbxo22*-KO mice. (A) Fertility testing of WT (n = 6), *Fbxo22*-sKO (n = 4), *Fbxo22*-gKO (n = 4), and *Fbxo22*-KO (n = 5) mice. (B) Gross morphology of the testes of WT, *Fbxo22*-sKO, *Fbxo22*-gKO, and *Fbxo22*-KO mice. (C) Testis/body weight ratios of WT (n = 4), *Fbxo22*-sKO (n = 3), *Fbxo22*-gKO (n = 4), and *Fbxo22*-KO (n = 4) mice. (D-F) CASA results for the sperm counts (D), motility (E), and progressive ratios (F) in WT, *Fbxo22*-sKO, *Fbxo22*-gKO, and *Fbxo22*-KO mice. M, million. n = 3 for each group. (G) H&E staining of sperm cells in the cauda epididymides of WT, *Fbxo22*-sKO, *Fbxo22*-gKO, and *Fbxo22*-KO mice. Scale bar = 20 μm. (H) Quantification of (G). n = 3 for each group. n.s., not significant.

to as *Fbxo22*-gKO and *Fbxo22*-sKO, respectively) mice (**Figure 3B**). Subsequently, *Fbxo22*-gKO mice were backcrossed with WT mice to produce heterozygous mice, and heterozygous mice were intercrossed to obtain *Fbxo22* global knockout (referred to as *Fbxo22*-KO) mice (**Figure 3C**). PCR amplification was performed to confirm the genotypes of both WT and mutant mice (**Figure 3D**). Thus, we successfully established *Fbxo22*-cKO/KO mouse strains.

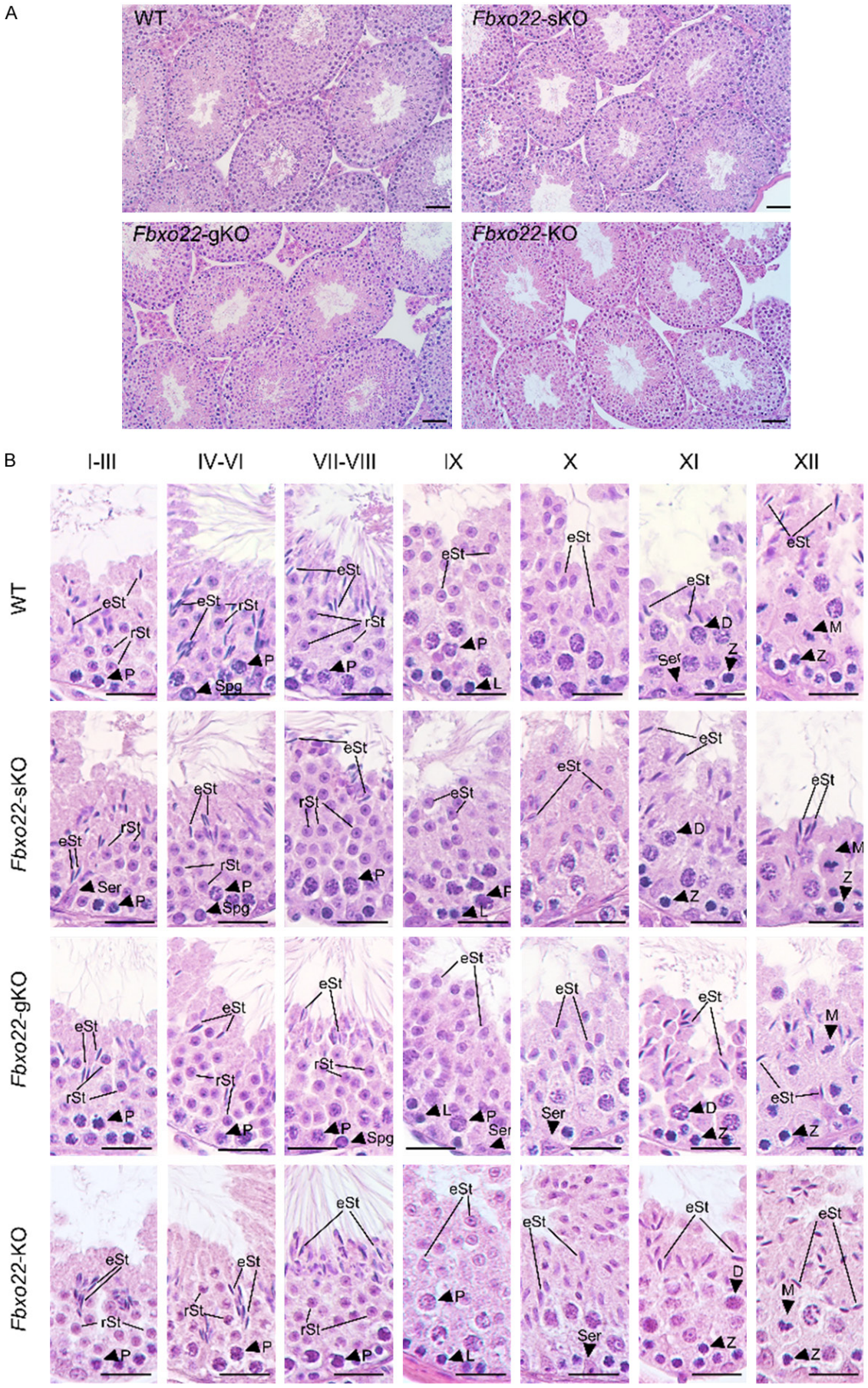
A
WT
Fbxo22-sKO
Fbxo22-gKO
Fbxo22-KO
B
I-III
IV-VI
VII-VIII
IX
X
XI
XII
WT
Fbxo22-sKO
Fbxo22-gKO
Fbxo22-KO
eSt
rSt
P
Spg
L
Ser
D
Z
M

Figure 5. Histology of the testes. A. H&E staining of paraffin-embedded testicular sections from WT, *Fbxo22*-sKO, *Fbxo22*-gKO, and *Fbxo22*-KO mice. Scale bar = 50 μm. B. H&E staining of spermatogenic stages I-XII in the testes of WT, *Fbxo22*-sKO, *Fbxo22*-gKO, and *Fbxo22*-KO mice. Scale bar = 25 μm. Ser, Sertoli cells; Spg, spermatogonia; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes; M, meiotic divisions; rSt, round spermatids; eSt, elongating/elongated spermatids.

Fbxo22-cKO/KO mice were fertile

Fbxo22-cKO/KO mice were viable and exhibited normal growth. Fertility tests revealed similar litter sizes compared to WT mice (**Figure 4A**). Testis size and weight were not significantly different between WT and *Fbxo22*-cKO/KO mice (**Figure 4B** and **4C**). Sperm analysis using CASA indicated normal concentration, motility, and progressive ratios in *Fbxo22*-cKO/KO mice compared to WT mice (**Figure 4D-F**). Additionally, sperm morphology evaluation via H&E staining revealed no significant differences between WT and *Fbxo22*-cKO/KO mice (**Figure 4G** and **4H**). Overall, *Fbxo22* was determined not to be essential for either fertility or semen quality in mice.

Fbxo22-cKO/KO mice show normal spermatogenesis

Testicular morphology was assessed by H&E staining. Both WT and *Fbxo22*-cKO/KO mice exhibited intact seminiferous tubules containing all stages of spermatogenic cells (**Figure 5A**). Spermatogenesis is a cyclic process marked by the sequential development of germ cells. In mice, the seminiferous epithelium cycle comprises 12 stages identifiable through H&E staining. These stages are distinguished by the unique distribution patterns of germ cells and the nuclear morphology of spermatids. Based on the H&E staining results, no significant morphologic changes were observed in the dynamics of germ cell development throughout the spermatogenic cycle between WT and *Fbxo22*-cKO/KO mice (**Figure 5B**).

Furthermore, SOX9, 3β-HSD, LIN28, SCP3, and PNA signals were utilized to quantify the populations of Sertoli cells, Leydig cells, spermatogonial stem cells, spermatocytes, and acrosomes in both WT and *Fbxo22*-KO testes, respectively. The results revealed no differences in cell counts between *Fbxo22*-KO and WT mice (**Figure 6**). These findings indicated that loss of *Fbxo22* does not influence mouse spermatogenesis.

Discussion

Previous studies have shown that several members of the F-box family are involved in spermatogenesis. For instance, *Fbxo7* mutant mice were infertile due to the phagocytosis of developing spermatids by Sertoli cells in late spermiogenesis [21]. Mutation in the *Drosophila Fbxo7* ortholog, nutcracker (*ntc*), similarly led to male infertility due to germ cell death during cytoplasmic remodeling [16, 22, 23], suggesting the conservation of the *Fbxo7* requirement at this developmental stage. F-box-only protein 47 (FBXO47) is a regulator of the telomeric shelterin complex; knocking out *Fbxo47* in mice caused male infertility by preventing the formation of a complete synaptonemal complex. Moreover, the interaction of FBXO47 with telomeric repeat-binding factor 1/2 (TRF1/2) destabilized TRF2, leading to unstable telomere attachment and delayed progression through the bouquet stage [24].

In this study, we generated *Fbxo22* cKO/KO mouse strains by CRISPR/Cas9 technology. However, no changes were detected in either fertility tests or histological analyses. The reproductive parameter results indicated that *Fbxo22* is not required for spermatogenesis or male fertility. Currently, research findings suggest that numerous genes encoding ubiquitin enzymes expressed in the testis are not necessary for mouse fertility (e.g., ankyrin repeat and SOCS box containing 12 (*Asb12*) [25], *Asb15* [26], F-box and WD-40 domain protein 17 (*Fbxw17*) [27]). Our findings eliminate *Fbxo22* as a target for contraception and as a factor in male infertility, which can prevent redundant research efforts and save resources in other laboratories. Furthermore, these results can aid reproductive researchers in prioritizing target gene research and focusing on genes crucial for fertility.

The expression and distribution of *Fbxo22* in the testes in this study were based on the analysis of testicular single-cell RNA sequencing data. However, these data lack experimental validation. Therefore, in future work, further

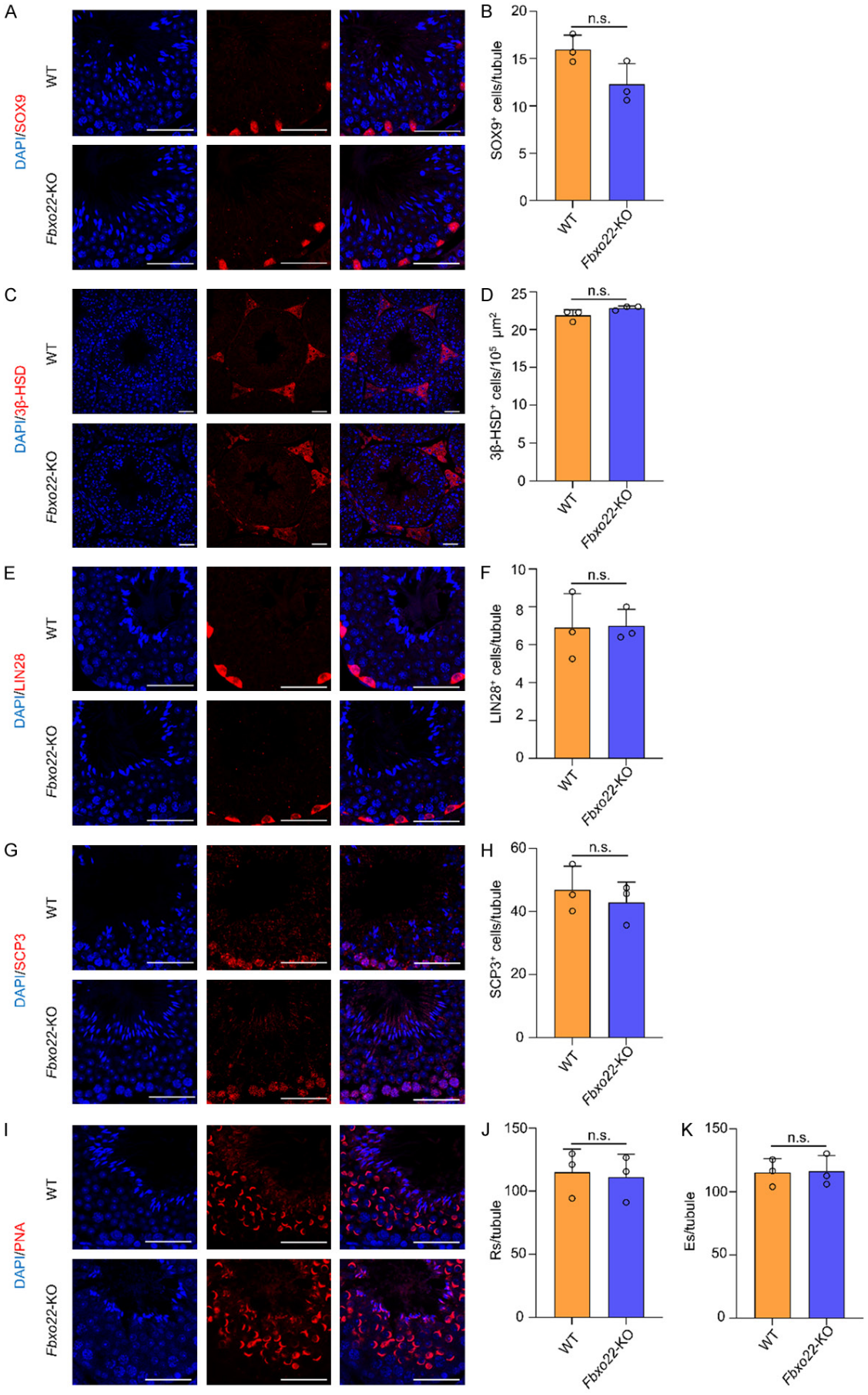
A
DAPI/SOX9
WT
Fbxo22-KO
B
SOX9⁺ cells/tubule
n.s.
WT
Fbxo22-KO
C
DAPI/3β-HSD
D
3β-HSD⁺ cells/10⁵ μm²
E
DAPI/LIN28
F
LIN28⁺ cells/tubule
G
DAPI/SCP3
H
SCP3⁺ cells/tubule
I
DAPI/PNA
J
Rs/tubule
K
Es/tubule

Figure 6. Immunostaining of stage-specific markers during spermatogenesis in WT and *Fbxo22*-KO mice. (A) Immunostaining of SOX9 in paraffin-embedded testicular sections from WT and *Fbxo22*-KO mice. Scale bar = 50 μm. (B) Quantification of (A). n = 3 for each group. (C) Immunostaining of 3β-HSD in paraffin-embedded testicular sections from WT and *Fbxo22*-KO mice. Scale bar = 40 μm. (D) Quantification of (C). n = 3 for each group. (E) Immunostaining of LIN28 in paraffin-embedded testicular sections from WT and *Fbxo22*-KO mice. Scale bar = 50 μm. (F) Quantification of (E). n = 3 for each group. (G) Immunostaining of SCP3 in paraffin-embedded testicular sections from WT and *Fbxo22*-KO mice. Scale bar = 50 μm. (H) Quantification of (G). n = 3 for each group. (I) Immunostaining of PNA in paraffin-embedded testicular sections from WT and *Fbxo22*-KO mice. Scale bar = 50 μm. (J) Quantification of round spermatids (Rs) in (I). n = 3 for each group. (K) Quantification of elongating/elongated spermatids (Es) in (I). n = 3 for each group. n.s., not significant.

clarification of FBXO22 expression and distribution in the testes can be achieved by preparing antibodies against FBXO22.

A plausible explanation for the normal spermatogenesis and fertility observed in *Fbxo22*-cKO and KO mice could be attributed to functional redundancy. Genetic compensation following gene knockout, where paralogous genes with overlapping functions step in to compensate for the loss of one gene, is a common phenomenon across species. For instance, the knockout of *Asb12* in mouse testes resulted in significant upregulation of *Asb-1*, *2*, *3*, *4*, *5*, *7*, *8*, *9*, *11*, *14*, *15*, *17*, and *18* [25]. Similarly, the loss of solute carrier 26a1 (*Slc26a1*) in mouse testes led to notable increases in the expression levels of *Slc26a5* and *Slc26a11* [28].

In summary, we generated *Fbxo22*-cKO and KO mice, which displayed normal spermatogenesis and fertility, revealing that *Fbxo22* is not significant for spermatogenesis and male fertility in mice.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32370903 to X.H. and 82301812 to D.Z.), the Gusu Health Talent Program of Suzhou (GSWS2020068 to B.Z. and GSWS2023011 to G.F.), the Key Research Foundation of Zhenjiang Social Development (SH2022029 to D.Z.), the Research project of Jiangsu Commission Health (Z2021048 to D.Z.), Medical Education Collaborative Innovation Foundation of Jiangsu University (JDY2023010 to D.Z.), the Suzhou Science and Technology Development Plan (SYS2020175 to G.F.), the Science and Technology Plan of Gusu School (GSKY20210203 to G.F.), the Suzhou Specialized Diagnosis and Treatment Techniques for Key Clinical Diseases (LCZX202013 to G.F.), and the Suzhou Youth Project of Science and Education for Medicine (KJXW2022030 to C.H.).

Disclosure of conflict of interest

None.

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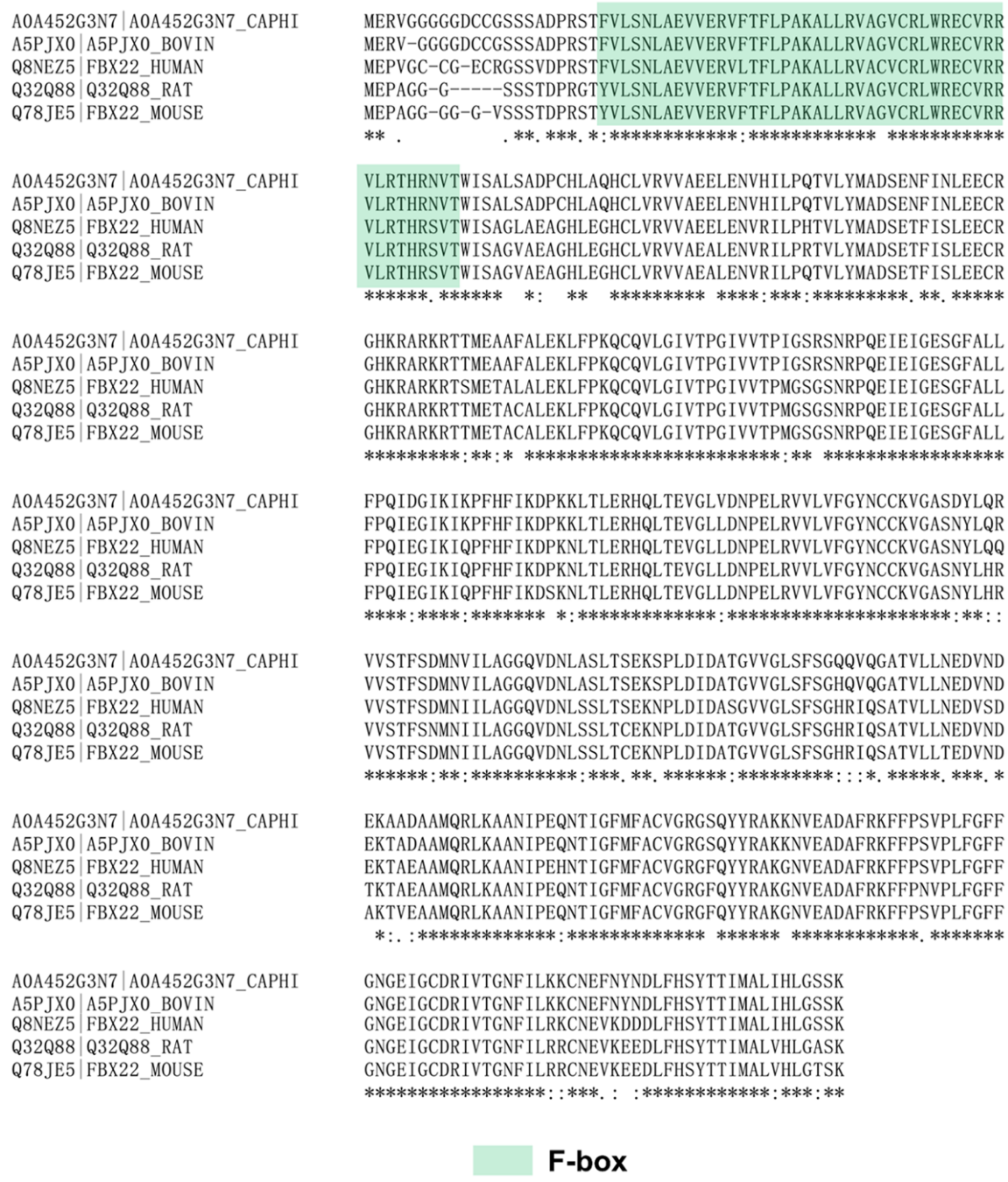

Supplementary Figure 1. Sequence alignment of FBXO22 proteins among species. Data are obtained from the Uniprot database (https://www.uniprot.org/).