Original Article

Testis-enriched *Asb12* is not required for spermatogenesis and fertility in mice

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Background: Members of the ankyrin repeat and SOCS box (Asb) family are expressed abundantly in testes. Some Asb genes/proteins are required for spermatogenesis, but the function of Asb12 during spermatogenesis is not clear. We investigated the physiological role of *Asb12* in murine testes.

Methods: The clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 system was used to generate *Asb12*-knockout (KO) mice. Histology and immunostaining were done to assess the effects of *Asb12* KO on mouse testes and epididymides. Semen quality was analyzed using a computer-assisted sperm analyzer. The terminal deoxynucleotidyl transferase-dUTP nick-end labeling assay was employed to examine testicular apoptosis. Real-time reverse transcription-quantitative polymerase chain reaction (PCR) was conducted to calculate gene transcription levels.

Results: *Asb12* was expressed predominantly in murine testes. Immunostaining of *Asb12* protein revealed that *Asb12* was located specifically in the acrosome of elongated spermatids, which suggested a potential role of *Asb12* during spermatogenesis. However, *Asb12*-KO mice had normal fertility, and no overt difference was detected in testicular morphology, semen quality, or apoptosis when comparing *Asb12*-KO and *Asb12*-wild type (WT) mice. Gene expression of several Asb family members was increased significantly in the testes of *Asb12*-KO mice when compared with that in *Asb12*-WT mice, which suggested functional compensation from paralogs for *Asb12* loss.

Conclusions: We demonstrated that *Asb12* is not essential for the spermatogenesis and fertility of mice. Our findings will assist researchers in avoiding redundant efforts, and provide a baseline resource for genetic studies on human fertility.

Keywords: *Asb12*; spermatogenesis; fertility; testis

Submitted Oct 11, 2021. Accepted for publication Jan 04, 2022.
doi: 10.21037/tau-21-900
View this article at: https://dx.doi.org/10.21037/tau-21-900

Introduction

To ensure the faithful transmission of genetic information to offspring, germ cells undergo dramatic dynamic remodeling throughout spermatogenesis. This process involves the self-renewal of spermatogonial stem cells (SSCs), meiosis, and spermatid maturation (1). Spermatogenesis is an intricate process and coordinated carefully by an elaborate network of genes (2,3). A total of 2,300 genes have been identified as testis-enriched genes in mice (4). Clarifying the role of these genes during spermatogenesis can reveal the molecular mechanisms underlying spermatogenesis, and assist in understanding the etiology of infertility and contraception in males. However, the function of many testis-enriched genes is not known.

Previously, using a gene-knockout (KO) approach mediated by the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system, we generated mice with deleted testis-enriched genes, and our data demonstrated some of them to be crucial for spermatogenesis. For example: T-complex-associated-testis-expressed 1 (TCTE1) is an important axonemal protein that is required for sperm motility (5); F-box protein 47 (FBXO47) is localized specifically in spermatocytes and directs integration between the telomere and inner nuclear envelope during meiotic progression (6); MRN complex interacting protein (MRNIP) is a MRN-interacting protein essential for chromosome synapsis and meiotic homologous recombination (7). On the other hand, we have also shown that several genes [Retinoic acid induced 14 (*Rai14*), testis expressed 33 (*Tex33*), Fibronectin type 3 and ankyrin repeat domains 1 (*Fank1*), and Eukaryotic elongation factor-2 kinase (*Eef2k*)], previously postulated as crucial factors for spermatogenesis, are dispensable for spermatogenesis and male fertility (8-11).

The ankyrin repeat and suppressor of cytokine signaling (SOCS) box (Asb) protein family contains 18 members in humans and mice. All of them have N-terminal ankyrin (ANK) repeats and a C-terminal SOCS box (12,13). ANK is required for substrate recognition, whereas the SOCS domain interacts with elongin B/C to mediate the ubiquitination and degradation of substrates (14). Recent evidence has revealed a definitive role for the Asb family during spermatogenesis. For example, *Asb3*, *Asb4*, *Asb8*, *Asb9*, and *Asb17* have been reported to be expressed abundantly in mouse testes (15-19); *Asb1*-KO mice exhibit hypospermatogenesis with an apparent loss of germ cells in seminiferous tubules (15); deficiency of *Asb17* in mice can prevent spermatogonial apoptosis through regulation of polyubiquitination of the anti-apoptotic proteins BCL2-like 2 (BCL2L2) and MCL1 apoptosis regulator, BCL2 family member (MCL1) (20). Apart from the roles of the Asb family during spermatogenesis, recent studies have indicated Asb members to be involved in development of the central nervous system (21), myogenesis (22), as well as tumorigenesis (23).

In the present study, we identified *Asb12* to be a novel testis-enriched gene in mice. We wished to uncover the role of *Asb12* in murine spermatogenesis using an *Asb12*-KO model. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-21-900/rc).

Methods

Ethical approval of the study protocol

This study was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (Bethesda, MD, USA). Animal experiments were approved (license number: 2004020) by the Animal Ethical and Welfare Committee of Nanjing Medical University (Nanjing, China).

Mice

Asb12-KO mice were generated using the CRISPR/Cas9 method according to a protocol reported previously by our research team (8,10). Single guide (sg)RNA was designed to target exon 4 of *Asb12*: 5'-GTAGATGCTACCACTAGGACAGG-3'. The target sequence was 5'-CCTGTCCTAGTGGTAGCATCTAC-3'. Generation of Cas9 mRNA was done as described previously (24,25). CD-1 mouse zygotes were co-microinjected with sgRNA and Cas9 mRNA. Founders were backcrossed with CD-1 mice to produce heterozygous mice. Pure-background CD-1 *Asb12*-KO and *Asb12*-wild-type (WT) mice were used for subsequent experiments. The genotype was identified by polymerase chain reaction (PCR) and subsequent Sanger sequencing using the forward primer 5'-AAGGAGGAGGAGGACACT-3' and reverse primer 5'-AGATGGAAGGTAGATGTTAGC-3'. Experimental animals were maintained under a specific pathogen-free condition at the Animal Center of Nanjing Medical University.

Fertility test

Three paired male (aged 8 weeks) *Asb12* (WT and KO) mice were caged, respectively, with WT females (aged 8 weeks) at a ratio of 1:2 for 2 months. The litter size and the number of pups born were recorded.

RNA extraction and real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from testicular tissues was extracted using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer instructions. Then, RNA was reverse-transcribed into complementary (c)DNA and processed using a real-time PCR system (Applied Biosystems, Foster City, CA, USA) for quantification. 18S rRNA served as an internal control. The primers for each gene are summarized in Table S1.

Histology

Testes or epididymides from *Asb12*-WT and *Asb12*-KO mice of age 8–12 weeks were collected and fixed in modified Davidson's fluid (MDF) for 48 h. Then, tissues were dehydrated in a graded series of ethanol solutions, embedded in paraffin, and sectioned at 4-μm thickness. These sections were rehydrated and stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS). Next, we undertook computer-assisted sperm analysis (CASA). Briefly, sperm from *Asb12*-WT and *Asb12*-KO mice aged 8–12 weeks were collected from the cauda epididymis and suspended in culture medium. Semen quality was assayed using the Ceros™ II Sperm Analysis System (Hamilton Thorne, Beverly, MA, USA). For ultrastructural analysis, sperm samples from *Asb12*-WT and *Asb12*-KO mice aged 8–12 weeks were fixed in 2% (*v*/*v*) glutaraldehyde overnight and immersed in 2% (*w*/*v*) OsO_4 for 2 h. Next, samples were embedded in araldite and sectioned at 80-nm thickness. Images were captured under a transmission electron microscope (JEM-1410; JEOL, Tokyo, Japan).

Immunofluorescence

Sections were deparaffinized and rehydrated, followed by antigen retrieval in sodium citrate buffer as described previously (26,27). After blockade with 1% (*w*/*v*) bovine serum albumin for 2 h, samples were reacted with the indicated primary antibodies (Table S2) overnight at 4 ℃. Nonimmune immunoglobulin (Ig)G antibodies were used for the negative controls (Figure S1). Subsequently, slides were washed thrice with phosphate-buffered saline (PBS), and then probed with Alexa-Fluor secondary antibodies (Thermo Scientific, Waltham, USA) for 1 h at 37 ℃. Finally, sections were counterstained with 4′,6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology, Nantong, China) and subjected to fluorescence analyses under a LSM800 confocal microscope (Zeiss, Oberkochen, Germany).

Terminal deoxynucleotidyl transferase-dUTP nick-end labeling (TUNEL) assay

Apoptosis was detected using a TUNEL kit (Vazyme, Nanjing, China) following manufacturer instructions. Briefly, paraffin sections were incubated with proteinase K for 15 min before being allowed to react with TUNEL labeling mix buffer for 1 h at 37 ℃. Apoptotic cells were counted under a confocal laser scanning microscope (LSM800; Zeiss).

Chromosome spread

Preparation of chromosome spreads was conducted as described previously (28). Briefly, testicular tissues from *Asb12*-WT and *Asb12*-KO mice aged 8–12 weeks were digested by collagenase and trypsin for 15 min at 37 ℃ before immersion in a hypotonic extraction buffer. Then, cell pellets were suspended in sucrose (100 mM) and spread on slides covered with fixative solution. After fixation, samples were washed with PBS and subjected to immunostaining with the indicated primary antibodies (Table S2).

Statistical analyses

Statistical analyses were conducted using Prism 8.0 (GraphPad, La Jolla, CA, USA). Data are the mean ± SD from at least three independent experiments. Biological replicates are indicated in the corresponding figure legends. The unpaired Student's *t*-test or chi-square test were used to calculate the differences between *Asb12*-WT and *Asb12*-KO mice.

Results

Generation of Asb12-KO mice

ASB12 belongs to the ASB protein family, in which all members have the classic ANK and SOCS domains. Bioinformatic analysis revealed ASB12 to be an evolutionarily conserved protein with conservation of the ANK and SOCS domains among species (Figure S2A). RT-qPCR revealed *Asb12* to be expressed abundantly in the mouse testis (Figure S2B). These findings suggested that *Asb12* might have a conserved role during spermatogenesis.

To explore the function of *Asb12 in vivo*, *Asb12* mutant mice were generated by CRISPR/Cas9-mediated gene editing which, ultimately, caused a frameshift mutation in exon 4 of *Asb12* on the X chromosome by a 1-bp insertion (*Figure 1A*). This mutation is predicted to result in a truncated protein of 123 amino acids, causing a partial loss of the ANK domain and complete loss of SOCS domains (*Figure 1B*). The mutation was confirmed by PCR and Sanger sequencing (*Figure 1C*). Furthermore, using immunostaining of the ASB12 protein in mouse testicular tissue and mature sperm, we observed an obvious location of ASB12 in the acrosome of *Asb12*-WT (+/Y) mice. In contrast, there was no detectable signal in the acrosome of *Asb12* mutant mice (*Figure 1D*,*1E*), suggesting an absence of the ASB12 protein in the testes of *Asb12* mutant mice. The fluorescence signal in the basal compartment of the seminiferous epithelium was probably non-specific because it was also present in the testes of *Asb12* mutant mice (*Figure 1D*). Thus, we first successfully constructed an *Asb12*-KO (–/Y) mouse strain. The specific location of ASB12 in mouse sperm suggested that ASB12 may be associated with acrosome maturation during spermatogenesis.

Normal fertility and semen quality in Asb12-KO mice

Asb12-KO mice developed and survived normally. *Asb12*-KO males were fertile and led to the production of similar litter sizes to that obtained with *Asb12*-WT mice (*Figure 2A*). The testes of *Asb12*-WT and *Asb12*-KO mice were comparable in size and weight (*Figure 2B*,*2C*). CASA revealed *Asb12*-KO mice to have a normal concentration, motility, and progressive ratio of sperm compared with those of *Asb12*-WT mice (*Figure 2D-2F*). H&E staining of sperm revealed *Asb12*-KO mice to exhibit normal sperm morphology compared with that of *Asb12*-WT mice (*Figure 2G*,*2H*). Due to the specific location of ASB12 in the acrosome of sperm, we undertook ultrastructural analyses of sperm in *Asb12*-WT and *Asb12*-KO mice. Ultrastructurally, *Asb12*-KO sperm displayed normal morphology with a well-formed acrosome, condensed head, and typical "9 + 2" axoneme (*Figure 2I*). Moreover, no significant change of progesterone-induced acrosome reaction between WT and KO was observed (Figure S3). We therefore concluded that *Asb12* was not required for fertility and semen quality in mice.

Normal spermatogenesis in Asb12-KO mice

Testicular morphology was examined by H&E staining. *Asb12*-WT and *Asb12*-KO mice exhibited intact seminiferous tubules with all stages of spermatogenic cells (*Figure 3A*). Moreover, there were no obvious histological changes of the epididymis between *Asb12*-WT and *Asb12*-KO mice (*Figure 3B*,*3C*). These data suggested that *Asb12* deficiency did not have an effect on spermatogenesis.

Spermatogenesis has three main processes: SSC (spermatogonial stem cell) proliferation, meiosis, and spermiogenesis. To determine precisely the process of spermatogenesis in the testes of *Asb12*-KO mice, we first undertook PAS staining to analyze the dynamics of acrosome development and nucleus morphology during spermiogenesis. There was no measurable morphological difference in spermatids between *Asb12*-WT and *Asb12*-KO mice during spermiogenesis (*Figure 4A*). In addition, the number of round spermatids and elongating/elongated spermatids was comparable between *Asb12*-WT and *Asb12*-KO mice (*Figure 4B*,*4C*). Moreover, Lin28, SOX9, and TUNEL signals were used to quantify the number of SSCs, Sertoli cells, and apoptotic cells in testes, respectively: the number of these cell types did not differ between *Asb12*-WT and *Asb12*-KO mice (*Figure 4D-4I*). Finally, the chromosome-spread method was conducted to evaluate meiotic progression. Under co-immunostaining of SCP3 (a marker of the lateral element of the synaptonemal complex) and γH2AX (a marker for DNA double-strand breaks), the subdivided stages of meiotic prophase (leptotene, zygotene, pachytene, and diplotene) could be distinguished readily according to the dynamic distribution of SCP3 and γH2AX (6,7,28). However, marked morphological alteration of SCP3 and γH2AX was not observed in *Asb12*-KO spermatocytes when compared with that in the WT group (*Figure 4J*,*4K*). Taken together, these results indicated that *Asb12* was dispensable for mouse spermatogenesis.

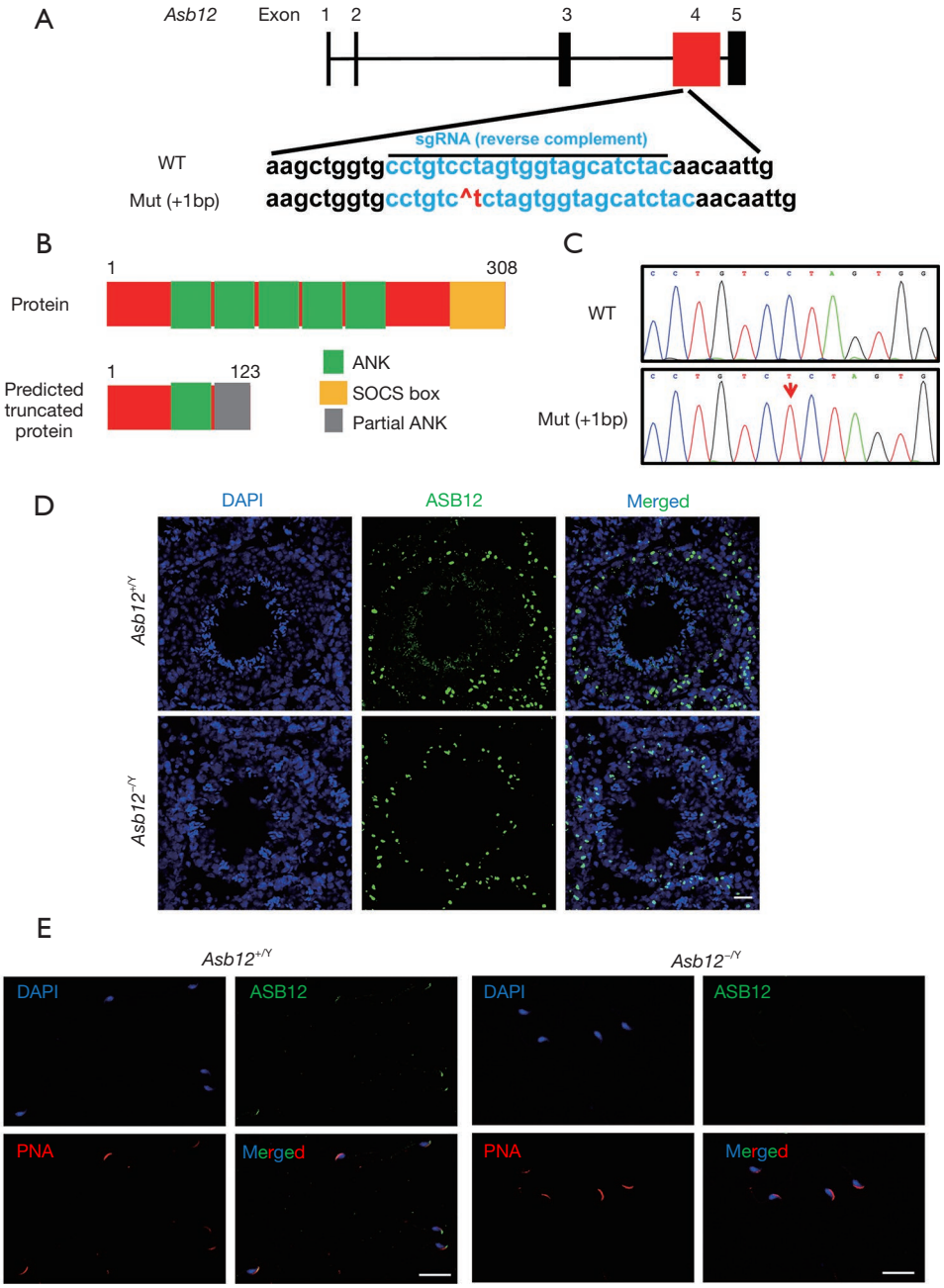

Figure 1 Generation of *Asb12*-KO mice. (A) CRISPR/Cas9-mediated *Asb12* editing (schematic). (B) Analysis of truncated protein in *Asb12*-KO mice. (C) Sanger sequencing of *Asb12*-WT and *Asb12*-KO mice. (D) Immunostaining of *Asb12* in the testes of adult *Asb12*-WT (+/Y) and *Asb12*-KO (–/Y) mice. Scale bar, 20 μm. (E) Immunostaining of *Asb12* in the sperm of *Asb12*-WT and *Asb12*-KO mice. PNA served as an acrosome marker. Scale bar, 20 μm. *Asb12*, Ankyrin repeat and SOCS box protein 12; WT, wild type; Mut, mutation; DAPI, 4′,6-diamidino-2-phenylindole; PNA, Peanut agglutinin.

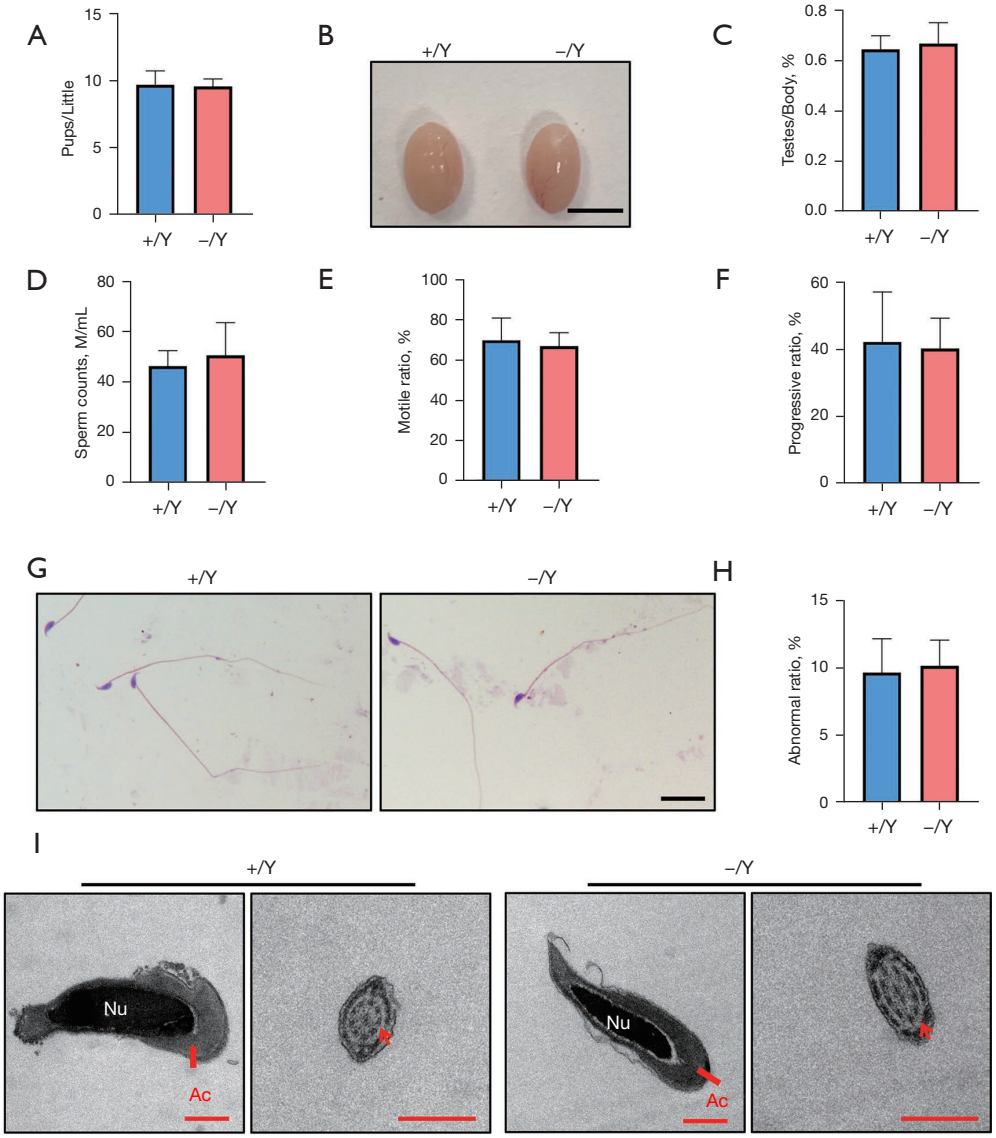

Figure 2 Normal fertility in *Asb12*-KO mice. (A) Fertility test of *Asb12*-WT and *Asb12*-KO male mice. n=3 for each group. P>0.05. (B) Gross morphology of the testes of *Asb12*-WT and *Asb12*-KO mice. Scale bar, 5 mm. (C) Testis/bodyweight ratio. n=3 for each group. P>0.05. (D-F) CASA of the counts (D), motility (E), and progressive ratio (F) of sperm in *Asb12*-WT and *Asb12*-KO mice. M, million. n=3 for each group. P>0.05. (G) H&E staining of sperm in the cauda epididymis of *Asb12*-WT and *Asb12*-KO mice. Scale bar, 20 μm. (H) Quantification of (G). n=3 for each group. P>0.05. (I) TEM of the sperm in the cauda epididymis of *Asb12*-WT and *Asb12*-KO mice. Arrows indicate axonemes with a "9 + 2" microtubule structure. Scale bar, 0.5 μm. Nu, nucleus; Ac, acrosome.

Functional compensation from paralogs in the testes of Asb12-KO mice

We undertook real-time RT-qPCR to assess the relative transcription levels of the 18 Asb genes in the testes of *Asb12*-WT and *Asb12*-KO mice. Interestingly, expression of *Asb-1*,*2*, *3*, *4*, *5*, 7, *8*, *9*, *11*, *14*, *15*, *17*, and *18* was increased significantly in the testes of *Asb12*-KO mice compared with that in the testes of *Asb12*-WT mice (*Figure 5*). The relative transcription level of *Asb12* was reduced markedly in the testes of *Asb12*-KO mice (*Figure 5*), which suggested that *Asb12* mutation could cause a decay of the encoded mRNA. This finding strongly suggests that other members of the Asb family may compensate for *Asb12* deficiency.

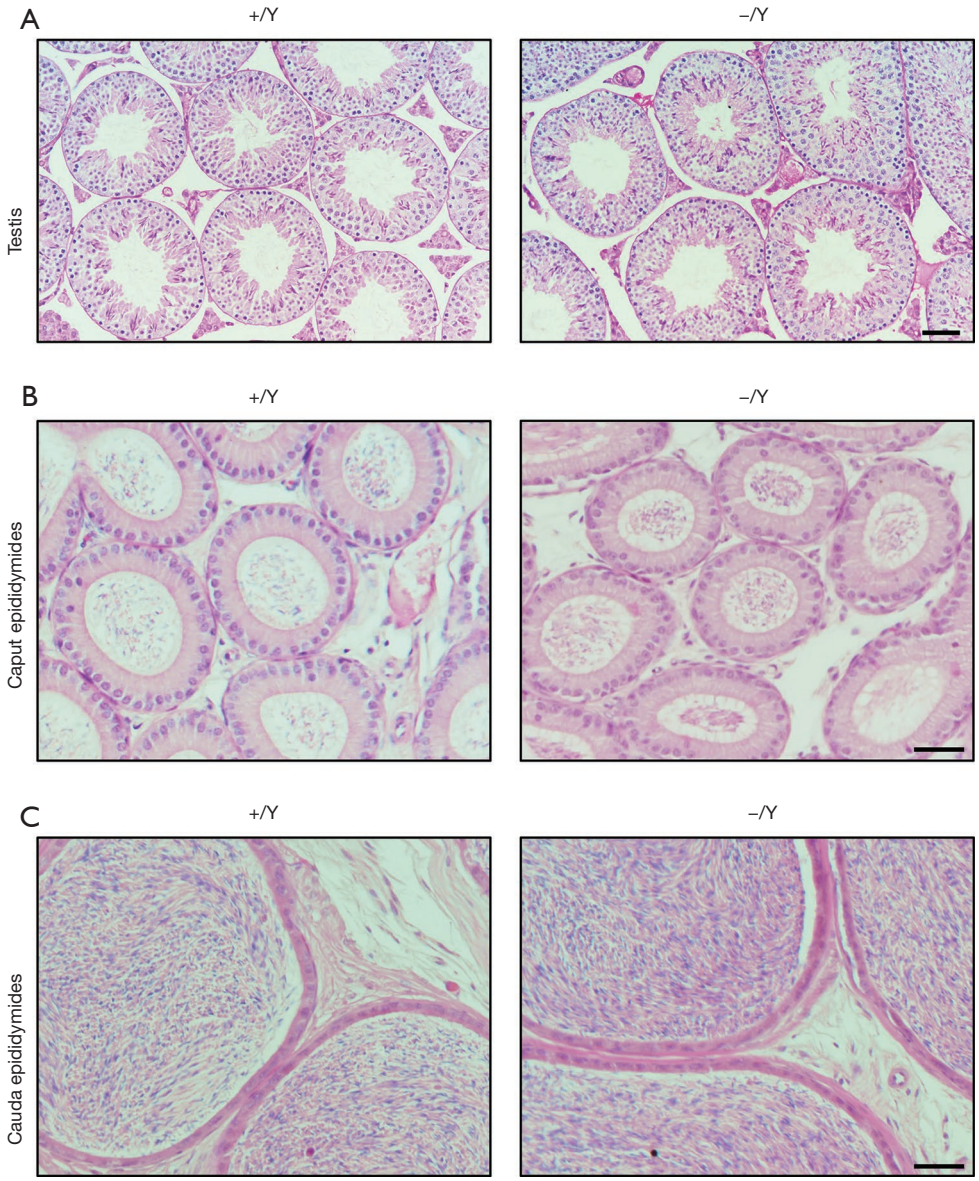

Figure 3 Histological analysis of testes and epididymides. (A) H&E staining of testis sections in *Asb12*-WT and *Asb12*-KO mice. (B) H&E staining of caput epididymides sections in *Asb12*-WT and *Asb12*-KO mice. (C) H&E staining of cauda epididymides sections in *Asb12*-WT and *Asb12*-KO mice. Scale bar, 50 μm.

Discussion

A gene microarray study by Schultz *et al.* uncovered >2,300 testis-enriched genes in the mouse genome. Using the gene-KO approach in mice, we and other research teams have revealed many testis-enriched genes that are required for spermatogenesis and male fertility (5-7,29-31). Nevertheless, several testis-enriched genes that had been postulated previously to be essential factors for spermatogenesis have been shown to be unnecessary for spermatogenesis in KO animal models (32). Although gene-KO mice with no obvious reproductive phenotypes have rarely been reported, the genetic and phenotypic information must be disseminated to the scientific community to prevent redundant efforts by other research teams. In 2017, Castaneda *et al.* shared information on 54 testis-enriched genes that are not essential for

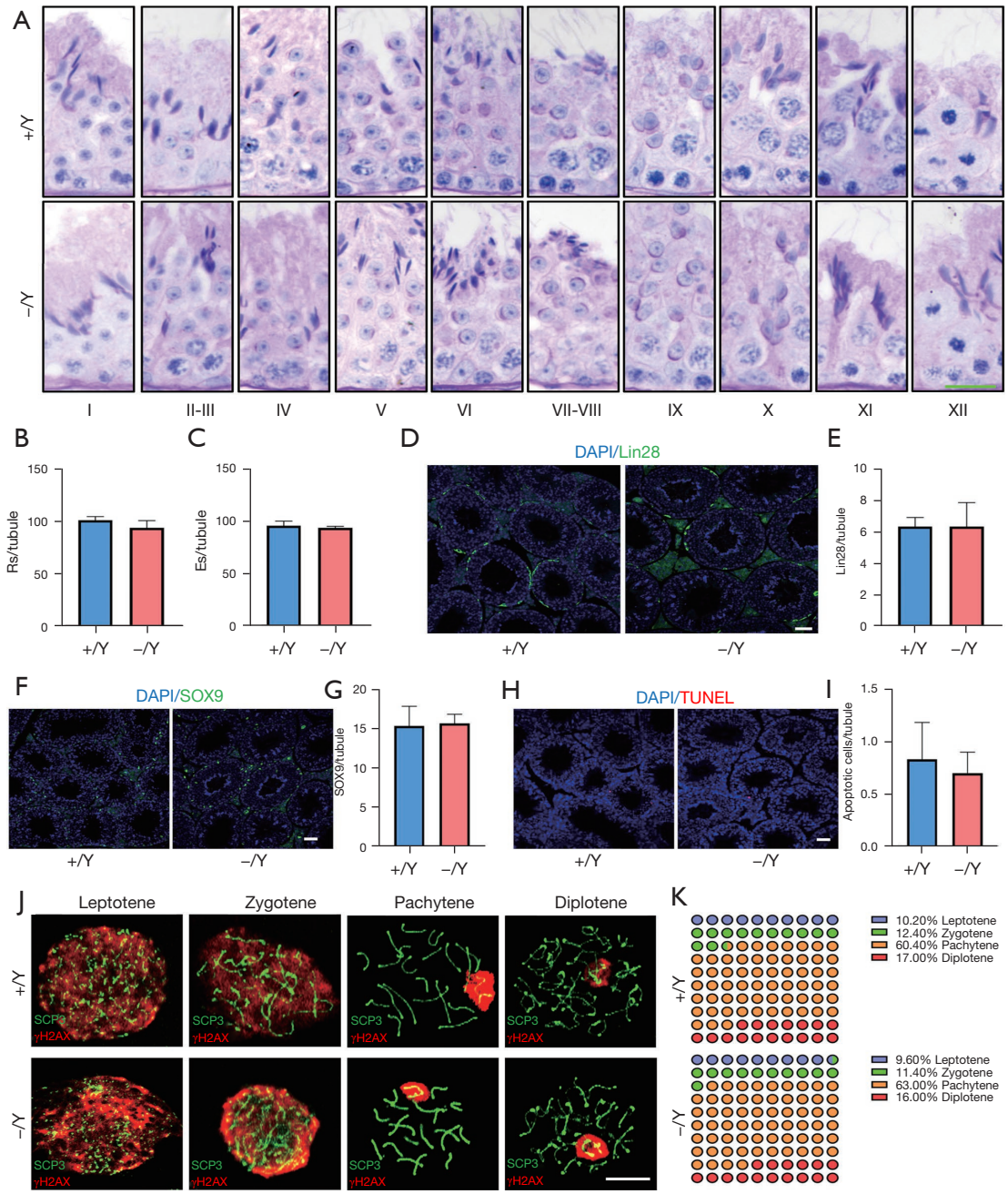

Figure 4 Normal spermatogenesis in *Asb12*-KO mice. (A) PAS staining of spermatogenic stages I–XII in the testes of *Asb12*-WT and *Asb12*-KO mice. Scale bar, 20 μm. (B) Quantification of round spermatids (Rs) in (A). n=3 for each group. P>0.05. (C) Quantification of elongating/elongated spermatids (Es) in (A). n=3 for each group. P>0.05. (D) Immunostaining of Lin28 in the testes of *Asb12*-WT and *Asb12*-KO mice. Scale bar, 50 μm. (E) Quantification of (D). n=3 for each group. P>0.05. (F) Immunostaining of SOX9 in the testes of *Asb12*-WT and *Asb12*-KO mice. Scale bar, 50 μm. (G) Quantification of (D). n=3 for each group. P>0.05. (H) TUNEL assay showing apoptotic cells in the testes of *Asb12*-WT and *Asb12*-KO mice. Scale bar, 50 μm. (I) Quantification of (H). n=3 for each group. P>0.05. (J) Co-immunostaining of SCP3 and γH2AX in the spread spermatocytes of *Asb12*-WT and *Asb12*-KO mice. Scale bar, 10 μm. (K) Distribution of stage-specific primary spermatocytes in (J). For each group, 500 spermatocytes were included. P>0.05. Rs, Round spermatids; Es, Elongating/elongated spermatids; DAPI, 4′,6-diamidino-2-phenylindole; Lin28, lin-28 homolog; SOX9, SRY-box 9; SCP3, synaptonemal complex protein 3; γH2AX, H2A.X variant histone; DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, Terminal deoxynucleotidyl transferase-dUTP nick-end labeling.

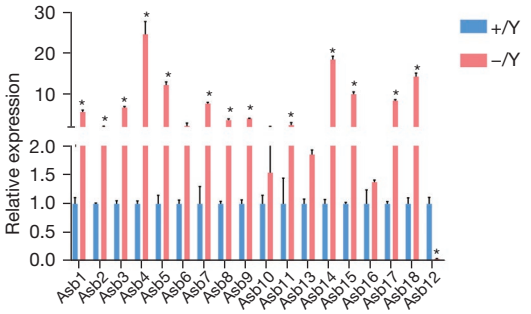

Figure 5 Functional compensation from paralogs in the testes of *Asb12*-KO mice. Real-time reverse transcription-quantitative polymerase chain reaction of the transcription levels of the 18 Asb genes in the testes of *Asb12*-WT and *Asb12*-KO mice. Gene expression was normalized to that of *18s rRNA*. n=3 for each group. *, P<0.05. Asb, ankyrin repeat and SOCS box.

spermatogenesis or fertility in mice via a genome-engineering approach. Thereafter, several large-scale screenings of non-essential testis-enriched genes were published (33,34).

Our research team has been using the CRISPR/Cas9 method to produce mice that delete testis-enriched genes. Here, we investigated the effects of the loss of a testis-enriched gene, *Asb12*, on spermatogenesis and fertility in mice using CRISPR/Cas9-mediated gene editing. We discovered that, despite the evolutionary conservation of *Asb12* in vertebrates, it is not essential for spermatogenesis and male fertility. Although such functional redundancy renders *Asb12* irrelevant as a target for contraception, the restricted expression of ASB12 in the sperm acrosome may enable it to serve as a potential biomarker for non-obstructive azoospermia.

A reasonable explanation for *Asb12*-KO mice displaying normal spermatogenesis and fertility may be functional redundancy. Genetic compensation upon gene KO is a common phenomenon among species. Paralogous genes with overlapping functions may compensate for the loss of one gene (35,36). For instance, KO of *Ubqln3* in mouse testes induces significant upregulation of expression of *Ubqln1*, *Ubqln2*, *and Ubqln4*, whereas KO of *UbqlnL* provokes a marked increase in expression of *Ubqln1* and *Ubqln4* in testes (37,38). Likewise, in our study, a loss of *Asb12* in mouse testes elicited a dramatic increase in expression of several Asb members. To overcome functional redundancy, generation of double-KO (or even triple-KO) models is needed to eliminate the effects of functional compensation from paralogs.

Conclusions

In this study, we generated a murine *Asb12*-KO model. Although *Asb12* was shown to be a testis-enriched gene in mice, *Asb12* itself had no obvious effects on spermatogenesis and male fertility in mice. The phenotypic information of *Asb12*-KO mice we have provided will assist other researchers in prioritizing their resources to focus on genes that are crucial for male fertility.

Acknowledgments

Funding: This work was supported by the National Natural Science Foundation of China (No. 81901532 to BZ), Natural Science Foundation of Jiangsu Province (No. BK20190188 to BZ, No. BK20190081 to ML), Open Fund of State Key Laboratory of Reproductive Medicine of Nanjing Medical University (No. SKLRM-K202010 to BZ), and Gusu Health Talent Program of Suzhou (No. GSWS2020068 to BZ).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-21-900/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-21-900/dss

Peer Review File: Available at https://tau.amegroups.com/article/view/10.21037/tau-21-900/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-21-900/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (Bethesda, MD, USA). Animal experiments were approved (license number: 2004020) by

the Animal Ethical and Welfare Committee of Nanjing Medical University (Nanjing, China).

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Cite this article as: Zhang R, Xu J, Shen C, Zhang X, Li S, Lv J, Xu D, Huang X, Zheng B, Liu M, Wu Y. Testis-enriched *Asb12* is not required for spermatogenesis and fertility in mice. Transl Androl Urol 2022;11(2):168-178. doi: 10.21037/tau-21-900

