ORIGINAL ARTICLE: XXX

TSPYL1 regulates steroidogenic gene expression and male factor fertility in mice

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Objective: To determine the importance of testis-specific, Y-encoded-like 1 (TSPYL1) in survival and male factor fertility in mice.
Design: Experimental prospective study.
Setting: Research laboratories in a university medical faculty.
Animals: We generated *Tspyl1* knockout (KO) mouse lines by CRISPR/Cas9. The lines were maintained by pairing heterozygous mice to provide wild-type control and KO males for comparison.
Intervention(s): None.
Main Outcome Measure(s): Mendelian ratio, body and testis weight, histology, sperm motility, mating tests, pregnancy outcome, transcript levels of genes for testosterone production, and serum testosterone level.
Result(s): A variable percentage of *Tspyl1* KO mice survived beyond weaning depending on the genetic background. Growth around weaning was retarded in KO mice, but the testes–to–body weight ratio remained normal and complete spermatogenesis was revealed in testis histology. Sperm was collected from the cauda epididymis, and a significantly smaller percentage of sperm was progressively motile (22.3% ± 18.3%, n = 14 samples) compared with wild type (58.9% ± 11.5%, 11 samples). All 11 KO mice tested had defective mounting behavior. From 11 KO males paired with a total of 88 females, only one litter was born, compared with 53 litters sired by 11 age-matched wild-type males. Expression of *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b6*, and *Hsd17b3* in the KO testis was significantly reduced, while serum testosterone level was within the normal range.
Conclusion(s): TSPYL1 is critical for survival and reproductive success in mice. TSPYL1 enhances the expression of key steroidogenic genes in the mouse testis. (Fertil Steril Sci® 2020;■:■–■. ©2020 by American Society for Reproductive Medicine.)
Key Words: TSPYL1, fertility, spermatogenesis, CYP, testosterone

Genes of the testis-specific, Y-encoded-like (TSPYL) family have been associated with male fertility in humans. There are 20–40 copies of *TSPY* (*Testis-specific, Y-encoded*) on the human Y chromosome (1). One study showed that there was a significantly higher number of *TSPY* copies found in infertile men compared with controls (2), while another study indicated that the copy number of *TSPY* has a positive correlation with sperm count, suggesting *TSPY* as a risk factor for male factor infertility (3). *TSPYL1* is located at 6q22.1. The gene is ubiquitously expressed (https://www.gtexportal.org/). A homozygous frameshift mutation in *TSPYL1* was identified in sudden infant death with dysgenesis of the testes (SIDDT) syndrome (4). Subsequently, heterozygous missense mutations in *TSPYL1* have been associated with 46,XY disorder of sex development and male factor infertility (5). In addition, an unreported rare variant that is likely pathogenic and another variant in *TSPYL1* have been identified in three spermatogenesis-impaired Han Chinese patients with a heterozygous status (6). Nevertheless, *TSPYL1* genetic variants do not seem to play a major role in male factor idiopathic infertility (7).

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TSPYL1 was identified as a novel gene with significant homology to *TSPY*, and it belongs to the nucleosome assembly protein (NAP) superfamily (8). The NAP superfamily consists of the three families of TSPYL, SET, and NAP1L1 according to the sequence conservation of the NAP domain. NAPs bind histones, and some members play a role in tissue-specific transcription regulation (9). There are five functional TSPYL genes that have no sequence conservation beyond the NAP domain. The X-encoded TSPYL2 inhibits the expression of androgen receptor target genes (10). TSPYL2 also regulates the expression of neuron-specific genes (11, 12) and is implicated in X-linked neurodevelopmental disorders (12–14). Transcription targets of other TSPYL genes remain largely unknown.

Sudden infant death syndrome is classified as the sudden death of an infant that occurs within the first year after birth, and the cause of death usually remains unknown even after a thorough postmortem autopsy (15). SIDDT syndrome is a specific lethal phenotype of sudden infant death syndrome that is characterized by cardiac or respiratory arrest with the dysgenesis of testes in male patients (Online Mendelian Inheritance in Man OMIM no. 608800). SIDDT was first reported in 21 infants from nine separate sibships among an Old Order Amish population and was attributed to homozygous loss of function of *TSPYL1* (4). Genotypic males with SIDDT had fetal testicular dysgenesis and ambiguous genitalia, with findings such as intra-abdominal testes, dysplastic testes, deficient fetal testosterone production, fusion and rugation of the gonadal sac, and partial development of the penile shaft. Female sexual development was normal. Nevertheless, TSPYL1 amino acid sequence variations were not related to sudden infant death in a German cohort (16) or deceased infants from Switzerland (17). Since SIDDT was reported only in an Amish community (4), rare variants of other genes from this community may be modifiers of the disease. Experimental evidence for the functional importance of TSPYL1 in testes development and male reproduction is greatly needed.

Recent studies show that TSPYL proteins regulate the expression of cytochrome P450 (CYP) genes, a large gene family encoding enzymes for steroid hormone biosynthesis and drug metabolism (18, 19). Specifically, a genome-wide association study shows that *TSPYL5* is associated with plasma estradiol level and that TSPYL5 induces *CYP19A1* expression in cell lines (18). CYP19A1 catalyses the synthesis of estrone and estradiol, whereas CYP17A1 is responsible for the biosynthesis of dehydroepiandrosterone and androstenedione. TSPYL1, TSPYL2, and TSPYL4 upregulate *CYP17A1* (19) as well as the serotonin transporter protein gene *SLC6A4* in cell lines (20). These data provide insight into how TSPYL1 variants may regulate steroidogenesis and brain function.

Here we take the approach of generating different strains of *Tspyl1* knockout (KO) mice to determine the significance of TSPYL1 in testes development and male fertility on different genetic backgrounds. We explore the relationship between TSPYL1 KO and transcript levels of genes involved in the testosterone biosynthesis pathway in our mouse models.

MATERIALS AND METHODS

Generation of *Tspyl1* KO Mice by CRISPR/Cas 9

Animals were kept in the Laboratory Animal Unit at the University of Hong Kong, a specific pathogen-free facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Mice were kept in M2 cages with 100 g Aspenchip bedding, with food and water available ad libitum, and maintained in a 22°C temperature-controlled barrier facility with light off from 7 p.m. to 7 a.m. At weaning, mice were segregated four to five per cage in single-sex groups. All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong (approval no. 4258-17).

The gRNAs with high target specificities were designed using the CRISPR Design Tool (http://tools.genome-engineering.org). The gRNAs were synthesized with MEGAshortscript T7 Kit (Thermo Fisher Scientific). The gRNAs and Cas9 protein (Sigma Aldrich) were injected into mouse zygotes by the Transgenic Core Facility at the University of Hong Kong. DNA samples were collected from ear clips at weaning, and polymerase chain reaction (PCR) was performed with primers 5'-GCCACTGCGATGCTGACA and 5'-CTTCTGCTCCACCTCCATCA. Successful introduction of *Tspyl1* mutations was identified by the loss of *Sac*II site or a smaller than wild-type PCR product and validated by sequencing. Two heterozygous male founders were obtained by using gRNA no. 1 (5'-TCACGAACACCGCTGCCGCG, *Sac*II site underlined followed by GGG in genomic DNA) in F1 (CBA/Ca X C57BL/6N) oocytes. A third heterozygous male founder was generated using gRNA no. 1 together with gRNA no. 2 (5'-TAGCTGCGGCCACCCGAATC) in C57BL/6N oocytes. Genotyping of this line was performed with primers 5'-GCTGCAAATGAGATCTTGGTTTCTG and 5'-GGGACCGT GAGGGTTGTTGTTTG. The two heterozygous male F1 founder mice were crossed with wild-type F1 (CBA/Ca X C57BL/6N) females, while the male C57BL/6N founder was mated with C57BL/6N females. Thereafter, the heterozygous offspring were used to maintain the line.

As litter size and other factors affect the growth of babies, littermates were used for collecting body weight and combined testicular weight. The genotype was not known at data collection.

Testis Histology

Testes of wild-type and age-matched controls were dissected out and fixed in 4% paraformaldehyde overnight. Subsequently, the testes were dehydrated in graded ethanol and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin using standard methods.

Sperm Motility Assay

The followings were performed blinded to genotype. Adult male mice were euthanized, and the cauda epididymis was dissected free from the surrounding fat. The epididymis was quickly ruptured using a 30 G needle at three points, and

epididymal sperm was gently squeezed out of the tubules in prewarmed medium 199 (Thermo Fisher Scientific). Samples were passed to a skilled investigator for rating motility. The sperm sample in 10 μL was placed on a prewarmed chamber glass slide (20 micron 2X-CEL sperm analysis chamber, Hamilton Thorne) and covered with a prewarmed coverslip. The samples were allowed to settle for 30–60 seconds to stop sperm drifting. Sperm motility was immediately examined under phase-contrast optics at ×200 magnification on a heated microscope stage at 37°C. The motility of the sperm was graded according to World Health Organization guidelines: grade A, progressive motility (spermatozoa moving actively, either linearly or in a large circle, regardless of speed); grade B, nonprogressive motility (all other patterns of motility with an absence of progression); grade C, immotility (no movement) (21). Multiple microscope fields were analyzed until around 200 spermatozoa were examined for each sample. The whole procedure was completed within 10 minutes of collection.

Fertility Tests

To quantify mounting activities, age-matched wild-type and KO males collected from the same breeding colony were used. Group housed males were separated to single cages 2 weeks before experiment. Institute of Cancer Research females of around 25 g were injected intraperitoneally with 7 units of pregnant mare serum gonadotropin (ProSpec) at 5 p.m., and 7 units of human chorionic gonadotropin (ProSpec) 47 hours later. Then one female was introduced to each male in the transparent 1155M cage and video was recorded with an IP-camera equipped with infrared night vision (Mi 1080P). The presence of vaginal plugs was checked the next day. The number of mounts for each male from 7 p.m. to 12 noon was counted from the videos. The test was performed and analyzed with the genotype blinded. Age and number of males were provided in the results.

Mating efficiency was further checked by natural mating. Wild-type and *Tspyl1* KO mice of 2.5–5 months old were used. Males of the same age were used in each cohort. The male mouse was caged with two fertile females for 16 days, with females were replaced every 4 days, that is, one estrous cycle. The presence of vaginal plugs was checked every morning. Successful pregnancy was scored when females gave birth and pups were counted.

RNA Extraction and Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was extracted from littermate samples using RNAzol RT (Molecular Research Centre). Two micrograms of RNA was reverse transcribed with SuperScript II (Invitrogen) using oligo-dT according to the manufacturer's instructions. Quantitative PCR was performed using QuantiFast SYBR green PCR kit (Qiagen) in a 9700HT Fast Real-Time PCR System (Applied Biosystems). The expression of genes was normalized to the expression level of the housekeeping gene *Hprt*. Duplicated reactions were conducted for each sample, and variations in threshold cycle (Ct) were within 0.2 cycle. The ΔΔCt method was adopted with relative expression to *Hprt* in wild-type being set as one (22). The sequences of primers in the 5' to 3' direction were as follows:

Tspyl1: CCCGCGGCAGCGGTGTTC; CTTCTGCTCC ACCTCCATCA

Cyp11a1: CTCAGTGCTGGTCAAAGGCTG; GTGCCC AGCTTCTCCCTGTAAA

Cyp17a1: TGGAGGCCACTATCCGAGAA; CACATGTGT GTCCTTCGGGA

Cyp19a1: TTCGCTGAGAGACGTGGAGA; CGACCTCTGG ATACTCTGCG

Star: AAACTCACTTGGCTGCTCAGTA; TGCGATAGGAC CTGGTTGAT

Hsd3b6: ACCATCCTTCCACAGTTCTAGC; ACAGTGACCC TGGAGATGGT

Hsd17b3: ATGGAGTCAAGGAGGAAAGGC; GGCGGTCT TGGTCATCTTGT

Hprt: AACTGGAAAGAATGTCTTGATTG; TCAAATCCA ACAAAGTCTGGC.

Measurement of Serum Testosterone

Serum samples were collected from adult male KO (n = 5) and control (n = 8) mice 3–6 months old, and the level of testosterone was measured using an ELISA kit (ADI-900-065, Enzo) according to the manufacturer's instructions at 1:10 and 1:20 dilutions. Testosterone (Sigma-Aldrich) was used to plot the standard curve.

Statistical Analysis

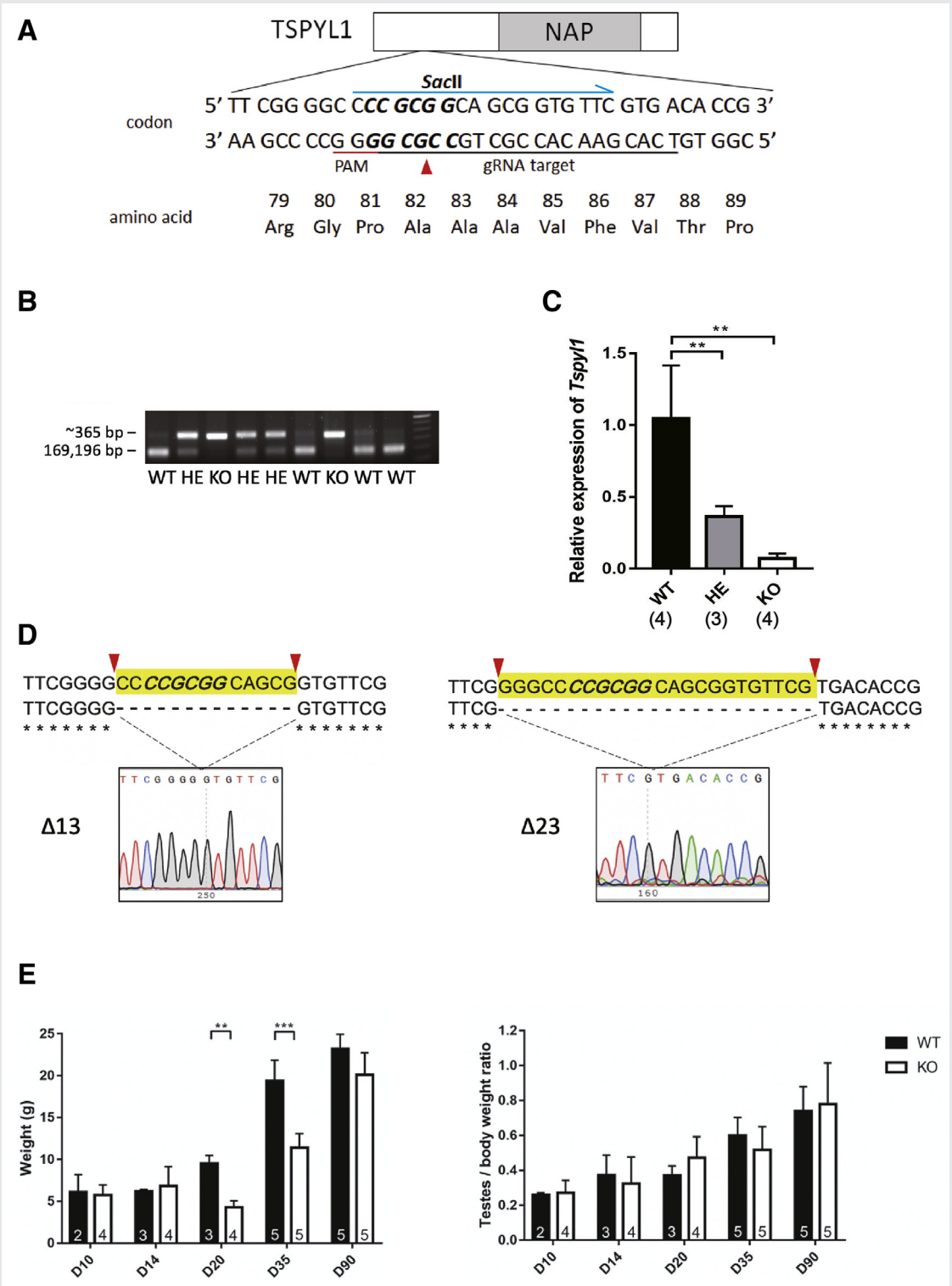
No statistical method was used to predetermine the sample size. GraphPad Prism 7.0 software (GraphPad Software) was used for statistical analyses. Chi-square analysis was used to detect any deviation from the Mendelian ratio. One-way analysis of variance (ANOVA) was used for comparison among three genotypes in RT-qPCR, and n equals the number of mice in each genotype. The sperm motility assay was analyzed by the Mann-Whitney test, and n equals the number of testis samples. Student's *t* test was used for comparison between wild-type and KO in other cases. The numbers of mice are indicated in figure legends or graphs. Assumptions of normal data distribution were adopted in Student's *t* test and one-way ANOVA. All statistical tests were two sided, and the data were presented as mean ± standard deviation. $P < .05$ was considered statistically significant.

RESULTS

Incomplete Penetrance of Lethality in *Tspyl1* Knockout Mice

Mouse TSPYL1 is made of 379 amino acids, and the NAP domain is at amino acid 173-351. The strategy was to target Cas9 to cut at codon 82, introducing an early frameshift mutation to cause loss-of-function mutations (Fig. 1A). The two mouse lines on the C57BL/6N mixed CBA/Ca genetic background were named Δ13 and in Δ23. Typical genotyping results by *Sac*II digestion of PCR products are shown in Figure 1B. Using a wild-type allele-specific forward primer (marked by a blue arrow in Fig. 1A), RT-qPCR validated

FIGURE 1



Generation of *Tspyl1* knockout mice. (A) Strategy to generate *Tspyl1* knockout mice. Nonhomologous end joining of deletions generated at the Cas9 cut site (arrowhead) also eliminates the *Sac*II restriction site before the coding sequence of the nucleosome assembly protein (NAP) domain. The blue arrow marks the sequence of the allele-specific forward primer for real-time quantitative polymerase chain reaction (PCR). (B) Genotyping results after digestion of PCR products with *Sac*II. (C) Relative expression of wild-type *Tspyl1* in adult testes. WT = wild-type; HE = heterozygous; KO = knockout. Transcription levels were normalized to *Hprt*. Mean ± standard deviation (SD), with the number of mice indicated in brackets. $^{**}P < .01$, one-way analysis of variance. (D) Sequencing results from homozygous mutants. (E) Reduced body weight at postnatal day 20 and 35 but normal combined testicular weight–to–body weight ratio in littermate KO mice (n indicated in graph). Data combined from Δ13 and Δ23 litters and presented as mean ± SD.$^{**}P < .01$ and $^{***}P < .001$, Student's *t* test.

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TABLE 1

Non-Mendelian ratios of pups derived from heterozygous parents.

Mouse lines	Δ13			Δ23			Δ463		
Genotype	+/+	+/Δ13	Δ13/Δ13	+/+	+/Δ23	Δ23/Δ23	+/+	+/Δ463	Δ463/Δ463
Observed (%)	27 (32.9)	46 (56.1)	9 (11.0)	67 (32.5)	109 (52.9)	30 (14.6)	72 (42.35)	96 (56.47)	2 (1.18)
Expected (%)	20.5 (25)	41 (50)	20.5 (25)	51.5 (25)	103 (50)	51.5 (25)	42.5 (25)	85 (50)	42.5 (25)
χ^2 value	9.12			13.99			60.49		
P value	.0105			.0009			< .0001		

Note: Genotyping was done at postnatal day 25–30. The expected number of wild-type (+/+):heterozygous:homozygous mice was calculated based on the Mendelian 1:2:1 ratio. The observed number of homozygous mice deviated significantly from the expected for all three mouse lines, χ^2 test.

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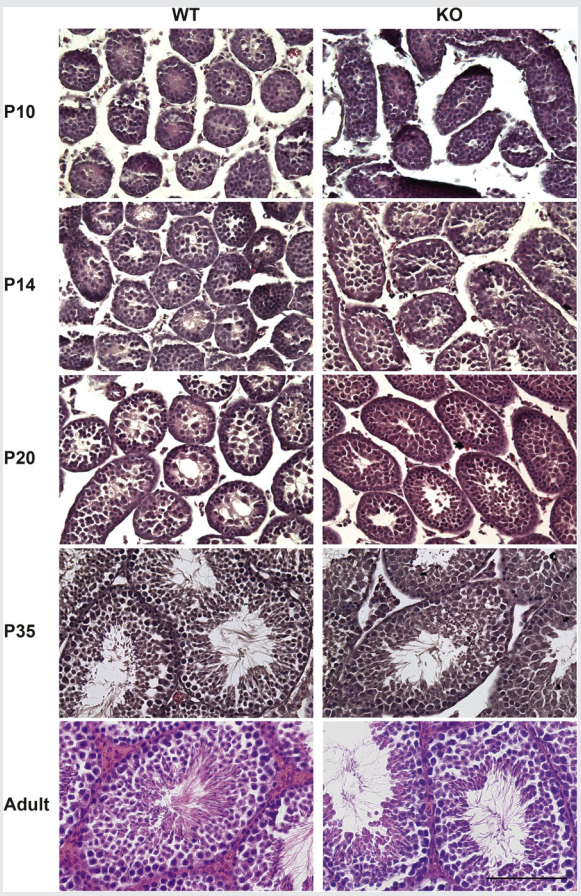
that levels of normal *Tspyl1* transcripts were reduced in heterozygous and homozygous (herein referred as KO) testes (Fig. 1C). Sequencing results of genomic DNA from KO mice confirmed a frameshift after proline 81 in Δ13 and after glycine 80 in Δ23 lines (Fig. 1D). We also generated one line with a 463 bp deletion on an inbreed C57BL/6N background and named it Δ463 (frameshift at codon 80). For all three lines, KO mice were indistinguishable from littermates during the first 2 weeks of life, but they appeared small and weak during days 18–22, which was around weaning time. Some KO mice died during this period, and autopsy did not reveal the possible cause of death. Genotyping at the time of death or at weaning confirmed that all three lines showed a deviation from the Mendelian ratio (Table 1). Almost all KO mice on the pure C57BL/6N background died around weaning. Therefore, only the two hybrid lines were used for the following study.

FIGURE 2



Normal postnatal testicular development in *Tspyl1* knockout mice. Representative hematoxylin and eosin–stained sections are shown. P = postnatal day; WT = wild-type; KO = knockout. Scale bar, 100 μm.

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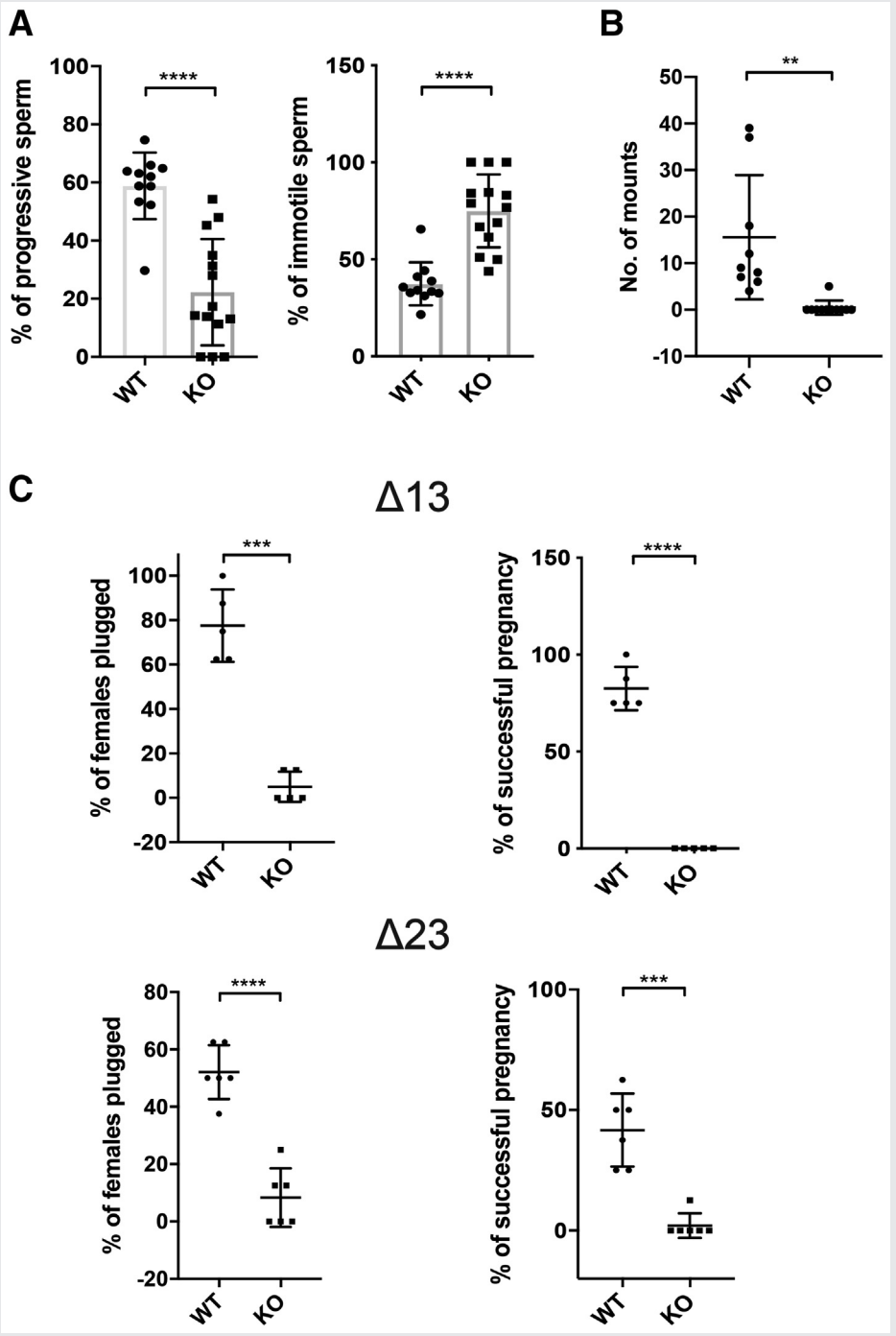
Surviving KO mice gradually resumed growth after weaning. At around 3 months, KO mice still appeared smaller, but the difference in body weight was not statistically significant (Fig. 1E, left). Despite the smaller body size or even early death, the testes–to–body weight ratio in KO mice was normal throughout postnatal development (Fig. 1E, right). Testis histology was also similar among littermates with the sequential appearance of early spermatocytes, pachytene spermatocytes, round spermatids, and elongated spermatids at postnatal days 10, 14, 20, and 35, respectively. Complete spermatogenesis was identified in histological sections of the adult KO testis (Fig. 2).

Poor Fertility of *Tspyl1* Knockout Males

Knockout males did not produce offspring after housing with fertile females for over 6 months. Therefore, we further investigated the reasons behind this. Experiments were conducted on age-matched animals when KO males were fully grown.

To access sperm motility, spermatozoa were collected from the cauda epididymis and graded according to the World Health Organization criteria (21). Sperm collected from each side of the mouse were treated as separate samples. In one KO mouse both sides had no sperm, and in one wild-type mouse there was no sperm from one side; therefore these samples were excluded for grading. In wild-type mice, there were higher percentages of grade A progressive motile sperm (mean, 58.9% ± 11.5%, n = 11 samples) than grade C immotile sperm (mean, 40.6% ± 10.9%); the reverse was true for KO (22.3% ± 18.3% grade A and 71.6% ± 17.4% grade C, n = 14 samples). Wild-type samples had a significantly higher percentage of progressive motile sperm compared with KO ($P < .0001$) and a significantly lower

FIGURE 3



Disruption of *Tspyl1* leads to male factor infertility in mice. (A) Reduced percentage of progressively moving sperms and increased percentage of immotile sperms from cauda epididymis of knockout mice. No. of samples = 11 wild-type (WT), 14 knockout (KO). Number of sperms rated in each sample = 180–262. Data are presented as mean ± standard deviation (SD). ****$P < .0001$, Mann-Whitney test. (B) KO mice rarely mounted receptive females. Each male was paired with an estrous female. Data on the total number of mounts during the first 5 hours of the dark cycle are presented. No. of samples = 9 WT, 11 KO. Mean ± SD, **$P < .01$, Student's *t* test. (C). The percentage of females with copulatory plugs (left) and giving birth (right) were significantly reduced for KO males. n = 5 males per genotype for the Δ13 line; six males for the Δ23 line; eight females per male. Data are presented as mean ± SD. ***$P < .001$ and ****$P < .0001$, Student's *t*-test.

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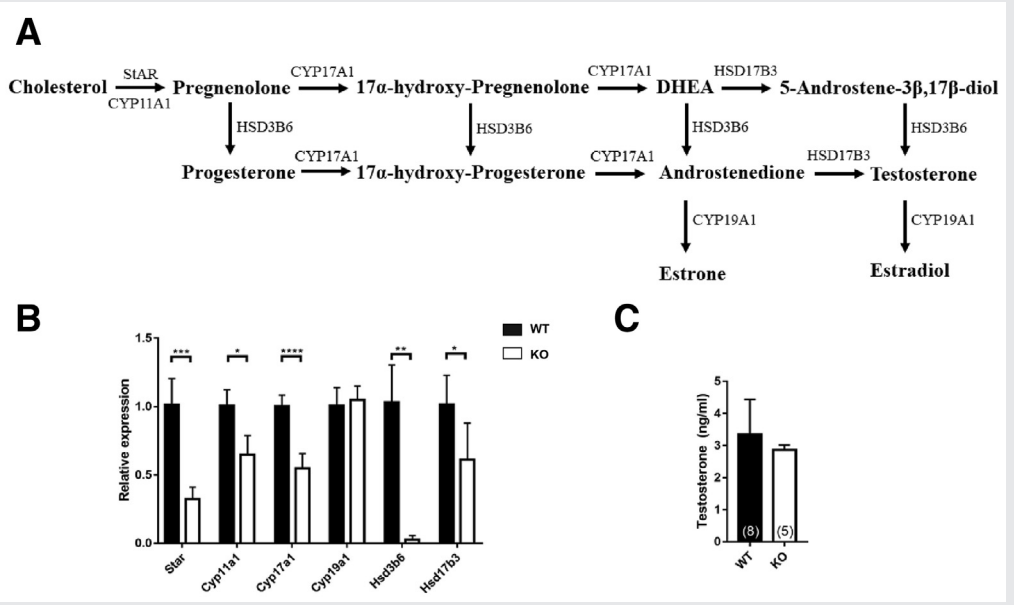
FIGURE 4



Tspyl1 knockout testis has reduced transcript levels of genes in testosterone biosynthesis. (A) Schematic diagram of steroidogenesis pathway in the mouse testis. (B) Relative expression levels of steroidogenic genes in adult wild-type (WT) and knockout (KO) testes (n = 5 mice each). Transcript levels of indicated genes were normalized to *Hprt*. (C) The serum testosterone level in *Tspyl1* knockout males is within the normal range (the number is indicated in brackets; $P = .2661$). Data are presented as mean ± standard deviation. *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$, Student's *t*-test.

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percentage of immotile sperms ($P < .0001$; Fig. 3A). The data indicated that a significantly smaller proportion of KO sperm was active.

Reduced percentage of motile sperm could not completely account for the dramatic reduction in fertility in all KO mice. To detect any abnormality in mating activities, we caged an adult male with an estrous female overnight and analyzed the video records. For Δ13 males, the test was performed at 5–6 months. During the first 5 hours of the dark cycle (i.e., 7 p.m. to 12 midnight), none of the four KO mice mounted, while all three control males mounted multiple times and the copulatory plug (coagulated semen) was identified in the vagina for two females the next morning. For Δ23 males, the experiment was performed at 3–4 months old. All six wild-type males had mounted multiple times, with copulatory plugs found in three females. For KO, only one out of seven KO males mounted, but no copulatory plug was found. The data of the two lines were combined and are shown in Figure 3B.

We further performed fertility assays using naturally ovulating females. Each male was allowed to mate with a total of eight fertile females over 16 days. Completed mating activity was recorded by the presence of a copulatory plug. For the Δ13 mouse line, the five wild-type males of 2.5–3 months old had plugged a total of 31 out of 40 females, with five to eight plugged females for each male (77.5% ± 16.3%). For the five KO males, there was only one plugged female each from two KO males (5.0% ± 6.8%, $P < .001$; Fig. 3C, left); for the Δ23 mouse line, the six wild-type males of 2.5–5 months had plugged a total of 25 of 48 females (three to five plugged females per male, 52.1% ± 9.4%), compared with a total of four plugged females from three KO males (8.3% ± 10.2%, $P < .0001$; Fig. 3C, left). The successful pregnancy rate was further reduced, with no females mated to Δ13 KO males giving birth, compared with 33 out of 40 females mated with wild-type males (six to eight females/male, 82.5% ± 11.2%, $P < .0001$; Fig. 3C, right). For the Δ23 line, only one female gave birth in the KO group, compared with 20 of 48 females (two to five per male) in the wild-type group (2.1% ± 5.1% vs. 41.7% ± 15.1%, $P < .001$; Fig. 3C, right). The age of the single KO father was 2.5 months old, and there were seven pups. The litter size was two to 12 for wild-type males 2.5–5 months old.

Reduced Transcription of Steroidogenic Genes in *Tspyl1* Knockout Testes Did Not Lower Serum Testosterone

TSPYL1 belongs to the NAP family, which can regulate transcription. Therefore, we investigated the expression of genes involved in the whole pathway of testosterone production, including *Star*, *Cyp11a1*, *Cyp17a1*, *Cyp19a1*, *Hsd3b6*, and *Hsd17b3* (Fig. 4A). The result from RT-qPCR indicated that there was a significant reduction in the relative expression of all the above genes except *Cyp19a1* in the testes of *Tspyl1* KO mice (Fig. 4B). When we measured the serum testosterone

level in adult mice, we found that the level in KO mice was within the normal range (Fig. 4C). Therefore, the reduction in the transcription of multiple steroidogenic genes did not have a significant impact on the level of serum testosterone in this case.

DISCUSSION

A homozygous loss-of-function mutation in *TSPYL1* has been identified in SIDDT patients from an Old Order Amish pedigree. However, this finding has not been replicated in other populations. We hypothesized that TSPYL1 is also important for regulating brain stem function and testis development in mice, and the effect is modified by the genetic background. To test this hypothesis, we generated several lines of mice with *Tspyl1* frameshift mutations using CRISPR/Cas9. Our data show that loss-of-function mutations in *Tspyl1* indeed cause growth retardation and premature death. Mutant mice on the mixed genetic background may recover from the growth retardation and present with infertility in adult life.

Since testes dysgenesis was not found in *Tspyl1* mutant mice, we investigated the possible causes of male factor infertility for animals that were able to recover from the growth problem. Our data show that impaired copulatory behavior in *Tspyl1* KO mice can account for the poor fertility of KO mice, while reduced expression of *Cyp17a1*, as well as other steroidogenic genes, is validated in our in vivo models, but this does not result in significantly reduced levels of circulating testosterone. Possible causes for the defects in mounting behavior include a defect during masculine differentiation of the limbic regions of the brain during development, which requires conversion of androgen precursors to estrogens (23, 24). In aromatase *Cyp19a1* KO male mice, there is impaired mounting (25). There might also be defective androgen receptor signaling in the nervous system, which regulates the extent of male-typical behavior (26). Furthermore, impaired androgen receptor signaling in the epididymis can lead to impaired sperm motility. In a mouse model with mutation of the androgen receptor SUMOylation site, spermatogenesis and serum testosterone level are unaffected but sperm motility and fertility are severely compromised, which are related to perturbed gene expression in the epididymis (27). Overall, our data revealed essential roles of TSPYL1 in sperm maturation and mating behaviors, but the molecular mechanisms remain to be determined.

The pleiotropic effect of TSPYL1 on growth and fertility may be due to the fact that TSPYL1 is ubiquitous and regulates gene expression in multiple regions of the brain. Besides controlling basic vital functions such as respiration, heartbeat, and sleep (28), the brain stem is connected to a range of hypothalamic and limbic system sites that play significant roles in the regulation of growth, emotion, and reproduction (29, 30). At present, we cannot rule out brain stem defects in *Tspyl1* mutant mice. Other limitations of our study include the high incidence of periweaning lethality, leading to difficulties in obtaining a larger number of KO mice for more powerful, detailed analysis. Off-target effects are also potential limitations of CRISPR/Cas9 technology. Our gRNA no. 1 is highly specific, and the highest-ranking potential off-target site in *Pask* (NM_080850) contains four nucleotide mismatches. We have sequenced this site in mutant mice and confirmed it to be normal (not shown). Furthermore, off-target mutations are unlikely to cosegregate with mutant *Tspyl1* and the phenotype. In our case, homozygous mice of our three mutant lines have the same phenotype of different expressivity.

Besides species difference in physiology, we wonder whether TSPYL1 has acquired additional functions in humans. When we aligned the human and mouse TSPYL1 amino acid sequences, 196 of 207 amino acids (95%) from the NAP domain to the short C-terminus are identical. However, the region N-terminal to the NAP domain is divergent in length and sequence, with 230 amino acids in humans and only 172 amino acids in mice. The findings that single nucleotide polymorphisms in the N-terminal region change the ability of human TSPYL1 in regulating *CYP3A4* and *CYP2C19* transcription (19, 20) could not be extended to mice.

CONCLUSIONS

The present study demonstrates that TSPYL1 is critical for body growth and survival around weaning and male fertility in mice. Knockout mice presented with normal testes development and poor growth before premature death and do not closely model patients of SIDDT. Mutant male mice that survive to adulthood rarely sire offspring, which can be ascribed to poor sperm quality and mating performance.

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