Male germ cell-specific knockout of cholesterogenic cytochrome P450 lanosterol 14α-demethylase (Cyp51)  

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Abstract  Cytochrome P450 lanosterol 14α-demethylase (CYP51) and its products, meiosis-activating sterols (MASs), were hypothesized by previous in vitro studies to have an important role in regulating meiosis and reproduction. To test this in vivo, we generated a conditional male germ cell-specific knockout of the gene Cyp51 in the mouse. High excision efficiency of Cyp51 allele in germ cells resulted in 85–89% downregulation of Cyp51 mRNA and protein levels in germ cells. Quantitative metabolic profiling revealed significantly higher levels of CYP51 substrates lanosterol and 24,25-dihydrolanosterol and substantially diminished levels of MAS, the immediate products of CYP51. However, germ cell-specific ablation of Cyp51, leading to lack of MAS, did not affect testicular morphology, daily sperm production, or reproductive performance in males. It is plausible that due to the similar structures of cholesterol intermediates, previously proposed biological function of MAS in meiosis progression can be replaced by some other yet-unidentified functionally redundant lipid molecule(s). Our results using the germ cell-specific knockout model provide first in vivo evidence that the de novo synthesis of MAS and cholesterol in male germ cells is most likely not essential for spermatogenesis and reproduction and that MASs, originating from germ cells, do not cell-autonomously regulate spermatogenesis and fertility.—Keber, R., J. Ačimovič, G. Majdić, H. Motaln, D. Rozman, and S. Horvat. Male germ cell-specific knockout of cholesterogenic cytochrome P450 lanosterol 14α-demethylase (Cyp51). J. Lipid Res. 2013. 54: 1653–1661.  

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The molecular mechanisms responsible for striking differences in the onset and progress of meiosis between sexes have been a matter of intense investigation. Early experiments suggested that a diffusible, sex-unspecific meiosis-inducing substance is produced by gonads of both sexes but induces meiotic initiation only in female germ cells. It was suggested that meiosis in the prespermatogonia could be blocked by the antagonistic action of meiosis-preventing substance, secreted by testicular cords (1,2). In 1995, two structurally related sterols, termed meiosis-activating sterols (MASs), with the ability to trigger the resumption of meiosis in mouse oocytes cultured in vitro, were identified (3). Follicular fluid meiosis-activating sterol (FF-MAS), isolated from human follicular fluid, and testis meiosis-activating sterol (T-MAS), isolated from bull testes, were the first reported sterol precursors with potential biological activity and the first compounds with meiosis-activating potency. Based on the sex- and species-unspecific action of MAS, a hypothesis about the important role of MAS in reproduction emerged (3,4). However, follow-up in vitro experiments have provided evidence that MAS might not be absolutely essential for gonadotropin-mediated resumption of meiosis (5). In contrast, some studies have demonstrated that MAS and related analogs promote nuclear and cytoplasmic maturation of oocyte in vitro (6). Therefore, despite the existence of a relatively large body of in vitro studies indicating an important regulatory role of MAS in the onset of meiosis in oocytes, in vivo proof has not yet been provided.  

Abbreviations: CYP51, cytochrome P450 lanosterol 14α-demethylase; 7-DHC, 7-dehydrocholesterol; DHCR24, 24-dehydrocholesterol reductase; DHL, 24,25-dihydrolanosterol; FF-MAS, follicular fluid meiosis-activating sterol; ko, knockout; LSS, lanosterol synthase; MAS, meiosis-activating sterol; qPCR, quantitative PCR; T-DHT, testosterone-dihydrotestosterone; T-MAS, testis meiosis-activating sterol; TM7SF2, transmembrane 7 superfamily member 2; wt, wild type.  
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†The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one figure and one table.
In contrast to oocyte, the role of MAS in male germ cell development has been poorly studied (7). Most of the clues indicating the potential role of MAS in spermatogenesis originate from the study of MAS-synthesizing cytochrome P450 lanosterol 14α-demethylase (CYP51), encoded by the gene Cyp51 in the mouse. CYP51 catalyzes the conversion of lanosterol and 24,25-dihydroxylanosterol (DHL) to FF-MAS and has recently been shown as essential for embryo development (8). FF-MAS is further converted to T-MAS by transmembrane 7 superfamily member 2 (TM7SF2) with sterol-Δ14-reductase activity. High accumulation of shorter testis-specific Cyp51 transcript was initially detected in humans (9). Detailed expression analyses of Cyp51 revealed the stage-specific expression of Cyp51 during spermatogenesis in rat (10). Low levels of Cyp51 transcript were first detected in the pachytene stage primary spermatocytes, whereas expression peaked in elongating spermatids and decreased thereafter. A similar expression pattern was also confirmed in mouse (11) and human testes (12). CYP51 protein was localized to the acrosomal regions of round and elongating spermatids, as well as residual bodies (13). Furthermore, isolated acrosomal membranes from ejaculated bull and bovine sperms were shown to transform lanosterol to FF-MAS (14). These results indicated that the regulation of Cyp51 in testes is different in male germ cells than in somatic cells and pointed to the possible functional role of cholesterol biosynthesis intermediates (FF-MAS and T-MAS) in spermatogenesis. One hypothesis suggested that MAS from residual bodies may influence the meiotic progression of spermatogonia, whereas the second hypothesis proposed that MAS from spermatoozoa may contribute to the completion of the second meiotic division of the oocyte during fertilization. To test directly the in vivo role of Cyp51 function and MAS produced in germ cells on spermatogenesis and male reproduction, we generated and characterized a male germ cell-specific knockout of Cyp51.

MATERIALS AND METHODS

Animals and sample processing

The generation of conditional gene-targeted Cyp51<sup>flox</sup>/<sup>flox</sup> mice (Cyp51<sup>fllox/</sup>, MGI: 4357830) and knockout (ko) Cyp51<sup>−/−</sup> allele (Cyp51<sup>−/−</sup>, MGI: 4836362) in C57BL/6 background was reported previously (8). The Cyp51<sup>flox</sup> allele contains loxP sites flanking essential exons 3 and 4 of the Cyp51 gene. In the ko allele, both exons are excised, resulting in a nonfunctional CYP51 enzyme causing lethality of homozygous Cyp51<sup>−/−</sup> embryos at E14.5. For the present study, Cyp51<sup>fllox/flox</sup> mice were bred to generate Cyp51<sup>−/−</sup> animals in which half of the gametes were already expected to carry a null allele. To delete Cyp51 in the postnatal premeiotic stages of male germ cell development in vivo, Cyp51<sup>fllox/</sup> mice were crossed to transgenic strain FVB/NJ-Tg(Stra8cre)1Reb/J (Stra8Cre<sup>+</sup>) obtained from The Jackson Laboratory (Bar Harbor, ME). Stra8Cre<sup>+</sup> mice carry a codon-improved CRE recombinase (15), driven by a promoter of the gene Stra8 (stimulated by retinoic acid 8), reported as having at least 95% efficiency of target gene excision in spermatogonia (16). These crosses yielded animals of two ko and two wild-type (wt) genotypes: Cyp51<sup>fllox/</sup>; Stra8Cre<sup>+</sup> (ko1), Cyp51<sup>fllox/flox</sup>; Stra8Cre<sup>+</sup> (ko2), Cyp51<sup>fllox/</sup>; Stra8Cre<sup>−</sup> (wt1) and Cyp51<sup>fllox/flox</sup>; Stra8Cre<sup>−</sup> (wt2). The mouse genotyping protocols were as described (8) or as recommended by The Jackson Laboratory for Stra8Cre<sup>+</sup> transgenic mice. Adult males at a minimum age of 10 weeks were used in all experiments and were euthanized with CO<sub>2</sub>. Testes were collected, weighed, and frozen in liquid nitrogen or used as described below. For histology, testes were fixed in Bouin’s fixative and processed for light microscopy by standard procedures. Three-micrometer sections were stained with hematoxylin and eosin (H and E). Plasma was collected using Li-heparin coated microtubes<sup>®</sup> (Sarstedt; Nürnbrecht, Germany). All animal experiments were performed in accordance with institutional and European directives for research animal use and were approved by the Veterinary Administration of the Republic of Slovenia.

Reproductive capacity

The reproductive capacity of males with a lack of function of Cyp51 in germ cells was tested in mating experiments and by measuring daily sperm production. Four littermate wt2-ko2 pairs of males were mated each with a pair of C57BL/6/OlaHsd females (Harlan; Udine, Italy). The number of litters and the number of offspring were documented for 57–103 days and evaluated in a statistical model using R software. The number of litters, number of offspring per litter, and the total number of offspring were compared between males of both genotypes and corrected for the number of days in mating. All pups were genotyped, and the number of offspring carrying allele Cyp51<sup>−/−</sup> was used to calculate the efficiency of Cyp51 gene excision in germ cells. Daily sperm production per gram of testes was evaluated as previously described (17). Briefly, testes were weighed and homogenized for 3 min in 25 ml of physiological saline containing 0.05% (v/v) Triton X-100 using polytron homogenizer T8.01 (IKA®-Werke; Staufen, Germany). Step 14–16 spermatids, which are resistant to homogenization, were counted twice at 100 × magnification to determine the average number of spermatids per sample. These values were used to calculate the number of spermatids per gram of testes, which was divided by 4.84 (the number of days that developing spermatids spend in steps 14–16) to obtain the efficiency of daily sperm production per gram of testes.

Isolation of germ cells by elutriation centrifugation

Mouse germ cell isolation was performed by a modified protocol (18). Briefly, pooled testes of two littermate males were decapsulated and finely minced with razor blades. Germ cells were released with vigorous pipetting in PBS, followed by filtration through a 100 μm cell strainer. The suspension was centrifuged for 5 min at 900 g. The cell pellet was resuspended in 20 ml of cold PBS and filtered through a 40 μm cell strainer prior to elutriation. The suspension was loaded into the JE-5.0 elutriation chamber (Beckman, CA), while the rotor was spinning at 2000 g and the PBS flowed at 5 ml/min. Next, the flow rate was incrementally increased up to 80 ml/min, and nine fractions of 100 ml were collected at each flow rate (7, 10, 15, 18, 23, 26, 30, 34, and 80 ml/min). Germ cell fractions were pelleted and frozen at −80°C for RNA and protein isolation. Aliquots of each fraction were transferred to a glass slide, fixed with 4% paraformaldehyde, and stained with 4',6-diamidino-2-phenylindole (Vector, CA). The proportion of different germ cell populations in individual fractions was evaluated with fluorescent microscopy.

Separation of seminiferous tubules and interstitial cells

The isolation of interstitial cells was done according to published protocol (19), with some modification. Briefly, testes were quickly removed, decapsulated, placed in M2 medium (Sigma; Mannheim, Germany) containing 0.2 mg/ml collagenase, and
incubated in a shaking water bath oscillating at 120 cycles/min at 34°C for 15 min. The dissociated cells were filtered and rinsed through a 100 μm cell strainer. The flow-through composed of interstitial cells was centrifuged at 1,000 g for 5 min and frozen at −80°C. Seminiferous tubules were washed from an inverted 100 μm cell strainer with PBS and centrifuged at 500 g for 5 min.

**Quantitative RT-PCR of genes in cholesterol synthesis**

Real-time quantitative PCR (qPCR) of cholesterogenic genes Cyp51, squalene epoxidase (Sqs), lanoster synthase (Lss), Tm7sf2, sterol-C4-methyl oxidase-like (Sc4mol), and 24-dehydro-cholesterol reductase (Dhc24) in germ cells and whole testes was performed on the LightCycler 480 detection system exactly as described (8). The expression of Cyp51 was quantified separately, using TaqMan® Assay-on-Demand gene expression assays (Applied Biosystems; Foster City, CA) positioned in excised exons 3 and 4. β-Actin was used as an internal control. Relative expression was calculated with the standard ΔΔCt method.

**Western blot analysis**

Total proteins were isolated using TRIzol® Reagent (Life Technologies; Darmstadt, Germany). Briefly, protein pellet was dissolved in 9 M UREA, 2% CHAPS for 1 h at 60°C, followed by 5× 1 min sonication at 20 kHz with 10 s cooling intervals. The total protein content was measured in diluted samples using the Bradford protein assay (Bio-Rad; München, Germany), and 80 μg of protein was loaded onto SDS-PAGE gels. Western blot analysis was performed as previously described (8). The anti-CYP51 antibody against mammalian-specific CYP51 peptide QRLKDSWAERLDFNPDRY was used in detection (GeneCust, Luxembourg). The signal on the membrane was visualized with the ultra-sensitive enhanced chemiluminescent substrate SuperSignal West Femto (Thermo Scientific; Rockford, IL).

**Measurements of cholesterol synthesis intermediates**

Quantitative analysis of nine cholesterol precursors and sterols was performed on whole testes, isolated seminiferous tubules, interstitial fractions, and plasma using a GC-MS method as described (20). The quantity of individual sterols was calculated and normalized to the wet weight of the testes and dry weight of the seminiferous tubules and the interstitial fraction.

**Measurements of plasma testosterone**

Testosterone levels were measured by radioimmunoassay (21). Because the anti-testosterone serum 250 showed very high cross-reactivity with dihydrotestosterone, the values are referred to as testosterone-dihydrotestosterone (T+DHT) levels. All samples were measured in triplicate (sensitivity 6 pg/tube; intra-assay coefficient of variation 5–8%).

**Statistical analysis**

The R statistical programming language (version 2.15.1) was used for statistical analyses. The Fligner-Killeen test of homogeneity of variances was used prior to conducting of parametric tests. One-way ANOVA was used with subsequent Tukey HSD posthoc tests to evaluate the differences between the two wt genotypes (wt1 and wt2) and the two knockout genotypes (ko1 and ko2). There was no significant difference (P<0.05) between the two wt groups and the two ko groups; therefore, statistical analysis was performed on the pooled wt group and pooled ko group. All statistics in the article present two sample t-test and Welch two-sample t-test (depending on homogeneity of variances) if not stated otherwise. Nominal P values of <0.05 were considered statistically significant.

**RESULTS**

**General characteristics of Cyp51 germ cell conditional null mice**

To provide in vivo evidence for a hypothesized role of Cyp51 and MAS in male germ cell development, we generated conditional germ cell-specific ko lines ko1 and ko2. Littermates lacking theCre transgene were used as controls in all experiments. The efficiency of germ cell-specific Cyp51 excision was tested using matings of ko2 transgenic males with C57BL/6OlaHsd females. The Cyp51+/− allele was detected in 112/126 offspring, indicating 89% efficiency of excision during spermatogenesis in the ko2 transgenic males. In ko1 males, in which every cell already carries one null allele, Cyp51 is expected to be lacking in 94.5% of spermatogonia, considering the aforementioned empirically determined 89% excision efficiency. High efficiency excision of Cyp51 locus was further confirmed by significant downregulation at the Cyp51 RNA and protein level using whole testes and separated germ cell fractions. Fractions containing primary spermatocytes (84% enriched), round spermatids (94% enriched), and elongated spermatids (97% enriched) (see supplementary Fig. I) were used. TaqMan expression analysis of excised exons 3 and 4 revealed 85–89% downregulation of Cyp51 expression in isolated germ cell fractions of ko animals (Fig. 1A). The expression of Cyp51 in whole testes of ko animals was reduced by 61% compared with wt littermate controls. Western blot analysis (Fig. 1B) demonstrated that only minute amounts of CYP51 protein were present in whole testes and elongated spermatids of ko2 animals, thereby confirming the Cyp51 mRNA analyses. The quantity of CYP51 protein in round spermatids was at the detection limit of the method used.

**Germ cell-specific Cyp51 inactivation affects cholesterogenic gene expression**

The expression of five genes from the cholesterol biosynthesis pathway was quantified in primary spermatocytes, round spermatids, elongated spermatids, and whole testes (Fig. 2). For the majority of tested genes, a trend of up-regulation was observed in the ko mice in primary spermatocytes and in round and elongated spermatids, but not in whole testes, where Leydig and Sertoli cells contribute to cholesterol synthesis. The sole statistical significance was displayed by the gene Lss, which was upregulated, albeit only by 1.3-fold, in the round spermatids of the ko mice. A trend of upregulating other cholesterogenic genes in the Cyp51 ko germ cells is probably a response to upregulate cholesterol biosynthesis, a phenomenon also observed in the previous full-knockout embryos (8).

**Germ cell-specific Cyp51 inactivation results in altered testicular sterol profiles**

The quantity of cholesterol, T-MAS, FF-MAS, and five other sterol intermediates was measured in whole testes, isolated seminiferous tubules, interstitial cells, and blood plasma of ko animals and wt controls (Fig. 3). At least four animals, age 10–12 weeks, were used per each genotype (ko and wt). Inactivation of Cyp51 in germ cells resulted in significant accumulation of immediate CYP51 substrates
did not influence the concentration of the end product, cholesterol, in the testes or isolated fractions (Fig. 3, middle panels). To evaluate blood as a possible source of sterol intermediates, we also quantified plasma using the same method. However, concentration of the four intermediates that were above the detection limit was very low and not significantly different between genotypes. Moreover, both MAS intermediates were below the detection level sensitivity of our method (Fig. 3D), confirming that the MAS observed in testes and their fractions did not originate in the blood. Concentrations of measured sterols are given in supplementary Table I.

Diminished function of CYP51 does not affect testicular weight and morphology, daily sperm production, or blood testosterone levels

To determine whether diminished function of CYP51 in germ cells affects the progression of spermatogenesis, we examined Bouin-fixed, H and E-stained sections of testes by light microscopy. Detailed histological analyses at different stages of the spermatogenic cycle revealed no differences between the seminiferous tubules of ko animals and wild-types. Germ cells of all maturation stages were present in the ko testes (Fig. 4A). Timing of the first spermatogenic wave was tested in 48 day-old males, revealing no delay in the onset of spermatogenesis in Cyp51 ko testes (data not shown). To further assess spermatogenic function, we analyzed daily sperm production with values corrected for testes weight. No significant differences in daily sperm production per gram of testes were detected between genotypes at the age of 12 weeks (Fig. 4B). Weights of testes with germ cell-specific inactivation of Cyp51 did not differ.
Male germ cell-specific Cyp51 knockout

Germ cell-specific Cyp51 loss of function does not affect reproductive performance in males

The reproductive performance of ko2 males was compared with that of wt2 littermates. Each of the four ko2-wt2 littermate pairs of males was kept with a pair of C57BL/6...
in targeted germ cells. These results confirm that excision frequency was among the highest when compared with other conditional ko models relevant for testicular physiology (22, 23). Male mice with germ cell-specific deficiency of Cyp51 did not display noticeable defects in testicular morphology, histology, or reproductive performance compared with wt males. Ko2 males yielded heterozygous Cyp51 +/− offspring in matings with C57BL/6 females, providing direct evidence that Cyp51-deficient sperm is able to fertilize an oocyte in vivo. We therefore conclude that lack of Cyp51 and/or MAS produced in male germ cells does not affect gametogenesis and fertility.

Successful conditional deletion of Cyp51 was demonstrated at the RNA, protein, and metabolite levels. In whole testes, the expression of Cyp51 mRNA was reduced on average by 61%. The remaining expression detected in the whole testes lysate is probably derived from nongerm somatic cells. Successful ablation of Cyp51 in germ cells was further demonstrated at the protein level. Only minute amounts of immunoreactive CYP51 protein were detected in elongated spermatids of ko animals (Fig. 1B). CYP51 was barely detectable in round spermatids (data not shown), confirming previously demonstrated stage-specific expression of CYP51 in germ cells (11). Germ cell-specific ablation of CYP51 was also supported by quantities and profile of sterol intermediates in the testes or its fractions. We detected high accumulation of CYP51 substrates

DISCUSSION

In the present study, we investigated the in vivo role of the lanosterol 14α-demethylase enzyme and its immediate products, meiosis-activating sterols in male reproduction, using a conditional Cyp51 knockout mouse model. The efficiency of Cyp51 inactivation in germ cells by spermatogonial-specific Stra8-Cre transgene was very high, as demonstrated by matings and Cyp51 allelic expression analyses in isolated germ cell fractions. Conditional excision efficiency of Cyp51 floxed allele during spermatogenesis in ko2 males was estimated at 89% and in ko1 males at 94.5%, as assessed in germ cell fractions. Considering that these germ cell fractions were enriched between 84% and 97% (see supplementary Fig. I) and hence could contain some contaminating somatic cells, we can claim that our model provided a nearly complete lack of Cyp51 function in targeted germ cells. These results confirm that excision frequency was among the highest when compared with other conditional ko models relevant for testicular physiology (22, 23). Male mice with germ cell-specific deficiency of Cyp51 did not display noticeable defects in testicular morphology, histology, or reproductive performance compared with wt males. Ko2 males yielded heterozygous Cyp51 +/− offspring in matings with C57BL/6 females, providing direct evidence that Cyp51-deficient sperm is able to fertilize an oocyte in vivo. We therefore conclude that lack of Cyp51 and/or MAS produced in male germ cells does not affect gametogenesis and fertility. Successful conditional deletion of Cyp51 was demonstrated at the RNA, protein, and metabolite levels. In whole testes, the expression of Cyp51 mRNA was reduced on average by 61%. The remaining expression detected in the whole testes lysate is probably derived from nongerm somatic cells. Successful ablation of Cyp51 in germ cells was further demonstrated at the protein level. Only minute amounts of immunoreactive CYP51 protein were detected in elongated spermatids of ko animals (Fig. 1B). CYP51 was barely detectable in round spermatids (data not shown), confirming previously demonstrated stage-specific expression of CYP51 in germ cells (11). Germ cell-specific ablation of CYP51 was also supported by quantities and profile of sterol intermediates in the testes or its fractions. We detected high accumulation of CYP51 substrates

**TABLE 1.** Reproductive capacity of males with a lack of function of Cyp51 in germ cells (ko) evaluated by matings

| No. of days in mating | No. of litters | No. of offspring | No. of ♀ offspring | No. of ♂ offspring |
|-----------------------|----------------|-----------------|-------------------|-------------------|
| 103                   | 8/10           | 46/51           | 20/19             | 26/32             |
| 91                    | 10/9           | 46/2            | 22/22             | 24/30             |
| 91                    | 7/8            | 48/53           | 22/25             | 26/28             |
| 57                    | 3/6            | 25/36           | 10/16             | 15/20             |
| **Average**           | **7/8.25**     | **41.25/48**    | **18.5/20.5**     | **22.75/27.5**    |

Fig. 4. Histology, daily sperm production, and testicular weight. A: Testes of males with conditional inactivation of the Cyp51 gene in germ cells (ko) do not display any changes in germ cell morphology compared with wild-type testes (wt), n = 4. B, C: There is no difference in daily sperm production (B) and testicular weight (C) between males of both genotypes. Bars indicate mean and SEM, n = 12–18.

Fig. 5. Concentrations of T+DHT in plasma of male mice evaluated by radioimmunoassay. No statistical difference was detected in T+DHT concentrations in males with conditional inactivation of Cyp51 in testes (ko) in comparison to wild-type animals (wt) at 10 (left) and 12 (right) weeks of age. Nonparametric Mann-Whitney-U test was used for statistical analysis (n = 10–17). The horizontal lines in the boxes represent the median and the lower and upper quartiles (25% and 75%). The 10th and 90th percentiles were used as whiskers. Upper and lower extreme values were plotted individually.
lanosterol and DHL when measured in the whole testes (Fig. 3A) and seminiferous tubules (Fig. 3B). DHL that is usually present in minute amounts in the wt testes was accumulated by 105-fold in the ko testes. The quantity of DHL in seminiferous tubules was below the detection level of our method. Significantly higher accumulation of DHL compared with lanosterol could result from upregulation of Dhcr24, transforming lanosterol to DHL, observed in other studies, where a single cholesterol biosynthesis gene was knocked out or its expression decreased (8). Our study shows that the genes in the cholesterogenic pathway display a trend of increased activity in germ cells, possibly to compensate for the lack of final product (cholesterol) and/or intermediate(s). It is known that genes involved in cholesterol synthesis are regulated coordinately by the sterol regulatory element-binding protein signaling pathway in somatic cells, whereas in male germ cells, alternate routes, such as cAMP/cAMP-responsive element modulator (CREM) τ, have been proposed (11). Our current data indicate that some sort of cholesterol feedback regulation might also be operating in germ cells.

In contrast to CYP51 substrates, the intermediates downstream of the CYP51 enzymatic step were significantly decreased in males with germ cell-specific Cyp51 deficiency. The levels of T-MAS, FF-MAS, desmosterol, and 7-DHC were decreased in whole testes and isolated seminiferous tubules. In testes and seminiferous tubules of ko animals, the quantity of T-MAS is decreased to roughly one-quarter of the levels in wt. If MAS serves a specific role in male germ cell development, as previously suggested, it is possible that only low amounts of T-MAS are already sufficient for full biological activity, and in such case, even a 70% decrease of T-MAS, as found in our study, could not be sufficient to interfere with the gametogenesis and fertility phenotype. The concentration of T-MAS in our wt mouse testes (40 µm) was in line with levels previously measured in mouse, bull, and stallion (>30 µg/g) testes (4). Comparisons of our measurements in seminiferous tubules are often not possible, inasmuch as the majority of previous studies determined T-MAS and other sterol intermediates in the whole testes or isolated sperm cells (4, 24, 25). In addition, previous studies have not addressed the quantity of sterols in the interstitial fraction, although a detected difference between high amounts of MAS in the whole testes versus low amounts in isolated sperm cells (4) indicated that interstitial cells might contain substantial amounts of MAS. Our results clearly demonstrate that interstitial fraction, primarily composed of Leydig cells and macrophages, accumulates significant quantities of sterol intermediates, similar to what we found in the germ cells. Sterol intermediates in interstitial fraction could serve as a backup resource of substrates needed to maintain stable synthesis of cholesterol for testosterone production. It is also possible that T-MAS and other sterol intermediates synthesized in the interstitial compartment of the testes are needed to perform a distinct function in spermatogenesis. Although specific mechanisms for the transport of sterol intermediates between somatic and germ cells in the testes have not yet been described, intermediates could be transported to seminiferous tubules along with the uptake of cholesterol.

Interestingly, Cyp51 deficiency did not affect the levels of cholesterol in the testes or isolated seminiferous tubules. This could indicate that cholesterol required for spermatogenesis mainly originates from sources other than germ cells. Early studies demonstrated that de novo synthesis of cholesterol in germ cells is not very efficient (26). The majority of nutrients for spermatogenesis, including lipids, are provided by the supporting Sertoli cells, which have the capacity to synthesize cholesterol from acetate in vitro (27). Because the cholesterol required to support spermatogenesis exceeds the biosynthetic capacity in Sertoli cells, cholesterol could be imported from circulation via specialized cholesterol transporters (28). Sertoli cells mainly depend on cholesterol acquired from HDLs by the apoE-dependent pathway (29, 30). The other important source of cholesterol might be the recycling of lipid-rich residual bodies and apoptotic germ cells, constantly phagocytized by Sertoli cells (31). Whatever the mechanism of compensation, we can conclude that germ cell-specific Cyp51 ko males are able to maintain wt levels of cholesterol for spermatogenesis, despite the blockage of de novo cholesterol biosynthesis in germ cells. Homeostatic mechanisms in charge of the import of cholesterol into the seminiferous tubule probably enable transport of other sterol intermediates along with cholesterol. It was demonstrated that the transport of some sterols from the site of synthesis to the plasma membrane is faster than that of cholesterol (32). The mobilization rate of different sterol precursors from the plasma membrane largely depends on the sterol structure (33). Interestingly, we detected a significant decrease of T-MAS in the interstitial fraction of ko males.

The origin and transport of sterol compounds in testes are very poorly understood (7), owing to the complexity of the system and the lack of highly sensitive methods to follow and quantify structurally similar lipids. More-detailed future studies of the dynamic sterol transport between the testicular cells are required to establish whether MAS is essential for spermatogenesis. If MAS is indeed involved in the meiotic progression of spermatogonia or completion of meiosis in the oocyte after fertilization, as previously suggested, it most probably originates from sources other than germ cells. Another explanation is that MAS function is taken over by other yet-unidentified molecules and/or redundant processes to compensate for the lack of MAS-producing CYP51 activity in germ cells. An example is the knockout of enzyme 24-dehydrocholesterol reductase (DHCR24), which catalyzes the final step in the synthesis of cholesterol. Dhcr24 knockout mice lacking cholesterol production that usually leads to embryo lethality are viable, inasmuch as desmosterol can functionally replace cholesterol (34). Alternatively, MAS may not have any biological role in spermatogenesis, and hence previous in vitro studies detected artificial or correlative associations that were not causal. However, our results unambiguously demonstrate that the presence of functional CYP51 in spermatozoa is not essential for fertilization, inasmuch as the Cyp51-deficient sperm maintained fertilizing ability in vivo.
It should be noted that in addition to MAS, other sterol intermediates were found to accumulate in the testes. For example, desmosterol is the predominant sterol intermediate in the testes of several mammalian species, including the mouse (35, 36), and sterol measurements in our study corroborate this. In some species, sterols such as lathosterol or cholesta-7,24-dien-3β-ol predominate in the testes (24, 37). The proportion of different sterol intermediates varies greatly by species and changes with the increasing age of animals. Some studies point to a role of intermediates (desmosterol) in promoting the motility of the sperm flagella (38). Desmosterol can replace cholesterol in cell membranes (39) and facilitates the membrane fluidity (40). We hypothesize that sterol intermediates, including MAS, are required to modulate the fluidity of sperm membranes during different stages of sperm development. According to this hypothesis, MAS does not serve a specific meiosis-activating role, as previously suggested. Late sterol intermediates have slightly different chemical structures and possibly different effects on membrane properties (41). A comprehensive picture of sterol-intermediate profiles in the testes is far from complete, because a full set of intermediates examined in all testes cell types and stages has not yet been systematically cataloged. Results from the present study demonstrate that perturbations of the cholesterol synthesis pathway in germ cells resulting in accumulation of pre-CYP51 intermediates (i.e., lanosterol and DHL) and decreased levels of post-CYP51 intermediates do not affect the reproductive capacity of males. It is plausible that owing to the similar structures of sterol intermediates, the function of an individual sterol is not absolute, resulting in the flexibility of the reproductive system. Such a system could provide high resistance to perturbations of sterol content mediated by diet or genetic factors, which is beneficial from the evolutionary standpoint. In conclusion, our germ cell-specific Cyp51 knockout model provides the first in vivo evidence that de novo synthesis of MAS and cholesterol in male germ cells is probably not essential for spermatogenesis and reproduction. Furthermore, our model demonstrates that Cyp51 and/or MAS from germ cells do not cell-autonomously regulate spermatogenesis and fertility in vivo. Our model carries a conditional Cyp51flox allele and therefore can provide material to other investigators for future tissue-specific conditional Cyp51 knockouts. Such studies can examine the role of cholesterol biosynthesis in particular organs/tissues, important for cholesterol homeostasis (i.e., liver) or organs in which de novo cholesterol biosynthesis is essential for proper development and function (e.g., brain, gonads).

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