Activin A, a product of fetal Leydig cells, is a unique paracrine regulator of Sertoli cell proliferation and fetal testis cord expansion

Denise R. Archambeaut and Humphrey Hung-Chang Yao

Department of Veterinary Biosciences, University of Illinois, Urbana, IL 61802

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Formation of tubular structures relies upon complex interactions between adjacent epithelium and mesenchyme. In the embryonic testes, dramatic compartmentalization leads to the formation of testis cords (epithelium) and the surrounding interstitium (mesenchyme). Sertoli cells, the epithelial cell type within testis cords, produce signaling molecules to orchestrate testis cord formation. The interstitial fetal Leydig cells, however, are thought only to masculinize the embryo and are not known to be involved in testis cord morphogenesis. Contrary to this notion, we have identified activin A, a member of the TGF-β protein superfamily, as a product of the murine fetal Leydig cells that acts directly upon Sertoli cells to promote their proliferation during late embryogenesis. Genetic disruption of activin A, the gene encoding activin A, specifically in fetal Leydig cells resulted in a failure of fetal testis cord elongation and expansion due to decreased Sertoli cell proliferation. Conditional inactivation of Snad4, the central component of TGF-β signaling, in Sertoli cells led to testis cord dysgenesis and proliferative defects similar to those of Leydig cell-specific activin A knockout testes. These results indicate that activin A is the major TGF-β protein that acts directly on Sertoli cells. Testicular dysgenesis in activin A and Snad4 conditional knockout embryos persists into adulthood, leading to low sperm production and abnormal testicular histology. Our findings challenge the paradigm that fetal testis development is solely under the control of Sertoli cells, by uncovering an active and essential role of fetal Leydig cells during testis cord morphogenesis.

In the majority of mammals, testis differentiation is initiated via expression of the sex-determining region of the Y chromosome (SRY) gene (1). Expression of both SRY and its related downstream target SRY-related HMG box gene 9 (Sry9) is restricted to somatic cell precursors that give rise to the epithelial Sertoli cell lineage (2). Sertoli cells are thus the first testis-specific cell lineage to arise in the XY gonad and the only cell type known to have a bias for the presence of the Y chromosome (3). Establishment of the Sertoli cell lineage is absolutely critical for testis morphogenesis, as it is considered capable of regulating all subsequent events in testis development (4). Sertoli cells direct testis morphogenesis by organizing testis cord formation, establishing testis vasculature, and inducing differentiation of other male-specific lineages including peritubular myoid cells and fetal Leydig cells (5). Sertoli cells work in concert with peritubular myoid cells to deposit a basal lamina that divides the testis into epithelial (testis cords) and mesenchymal (interstitial) compartments (6). Sertoli cells themselves align as the epithelium of the testis cords, with germ cells sequestered within the lumen. Interstitial fetal Leydig cells produce androgens and insulin-like factor 3 (INSL3) that masculinize the embryo and promote testis descent, respectively (7). However, fetal Leydig cells and their products are not known to influence testis cord morphogenesis.

Testis cords, embryonic precursors of the seminiferous tubules, arise as a complex series of parallel transverse loops separated by interstitial cells (8–11). Late in fetal development, testis cords undergo a poorly understood process of elongation and expansion that eventually leads to the convoluted structure of the seminiferous epithelium in the adult animal. Expansion of the Sertoli cell population, which continues into early postnatal life, is presumed to be critical for quantitatively normal sperm output in adulthood, as each Sertoli cell can support only a finite number of germ cells (12).

In many organs, development and maintenance of tubular structures requires cross-talk between epithelial and mesenchymal compartments. We became specifically interested in the potential involvement of activin A in fetal testis cord development because of its well-established roles in tubulogenesis within other tissues including kidneys, salivary glands, pancreas, prostate, Wolffian ducts, and dentition (13–16). Although compartmental specificity varies by tissue context, activin A often regulates epithelial patterning and development (17). Like other members of the TGF-β superfamily, activin A is a dimeric signaling peptide. Previous studies have shown expression of mRNA and protein for activin βA (a.k.a. inhibin βA or Inhba), the gene encoding activin A, in the interstitium of fetal mouse testes (18, 19). However, the function and cellular source of this interstitial activin A are not known. The best-characterized interstitial cell population is the steroidogenic fetal Leydig cells that, although critical for the development of androgen-dependent organs, are not known to be involved in testis morphogenesis. We therefore investigated the possibility that interstitial fetal Leydig cells might actively regulate testis cord development via activin A.

Results

Conditional Inactivation of Inhba in the Fetal Leydig Cells Results in Testis Cord Dysgenesis. To evaluate whether fetal Leydig cells are a source of interstitial activin A, we inactivated Inhba using the anti-Müllerian hormone type 2 receptor-cre (Amhr2-cre) mouse strain in which cre recombinase activity is targeted specifically to fetal Leydig cells (20, 21). We conducted a time-course analysis of testis morphogenesis in Amhr2cre+;Inhba−/− conditional knockout (Inhba cKO) embryos to determine whether loss of fetal Leydig cell-derived activin A altered testis cord organization and/or maintenance. Immunohistochemistry for laminin was used to mark the basal lamina at the boundary of testis cords in transverse sections (Fig. 1 A–I). In both control (Amhr2cre+;Inhba+/+) and Inhba cKO embryos, testis cords appeared as transverse circular loops at embryonic day 15.5 or E15.5 (Fig. 1 A and B). The testis cords of control embryos began to coil at E17.5 (Fig. 1 D) and underwent further convolution by E19.5, the time of birth (Fig. 1 G), revealing numerous small cross-sections of the cords. Compared with controls, Inhba cKO

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1To whom correspondence should be addressed. E-mail: hhyao@illinois.edu.
testis cords at E17.5 exhibited fewer circular sections, indicative of reduced coiling (Fig. 1E). By E19.5, Inhba cKO testis cords underwent only minimal coiling, and their initial circular shape was still largely visible (Fig. 1F). At all stages analyzed, development of other reproductive organs was indistinguishable between control and Inhba cKO mice.

Disruption of testis cord morphogenesis in Inhba cKO embryos resulted in abnormal testis histology by birth. Sagittal histological sections from E19.5 control (Fig. 1J) and Inhba cKO (Fig. 1K) testes revealed a gross enlargement of testis cord cross-sectional diameter in Inhba cKO embryos. In addition, fewer testis cord cross-sections were visible in Inhba cKO testes compared with controls. Straight lengths of testis cords were seen emerging from the rete testes in Inhba cKO embryos because of severe deficits in coiling (Fig. 1K). Under higher magnification, occasional Sertoli cells were observed within the lumen of Inhba cKO testis cords (Fig. 1N, black arrow) but not in controls (Fig. 1M). In summary, the normal appearance of testis cord loops in Inhba cKO testes through E15.5 suggests that fetal Leydig cell expression of activin A is dispensable for initial testis cord formation and maintenance. However, the dramatic reduction in convolution of Inhba cKO testis cords thereafter indicates fetal Leydig cell-derived activin A is a critical regulator of testis cord expansion between E15.5 and birth.

Sertoli Cell-Specific Ablation of Smad4 Recapitulates Testis Cord Dysgenesis Phenotypes Found in Fetal Leydig Cell-Specific Inhba Knockout Embryos. To investigate whether fetal Leydig cell-derived activin A signals to the Sertoli cell epithelium, we used the anti-Müllerian hormone-cre (Amh-cre) transgenic mouse line to remove Smad4 within the Sertoli cell epithelium (22, 23). As the central component of canonical TGF-β superfamily signaling, deletion of SMAD4 should eliminate canonical TGF-β signaling to the Sertoli cells (24). Although numerous TGF-β superfamily ligands, including AMH, TGF-β1, TGF-β2, TGF-β3, and activin B are produced in embryonic testes, testis cord dysgenesis has not been reported in murine models lacking these genes (19, 25–27). We therefore hypothesized that any changes in testis cord development in the Sertoli-specific Smad4 conditional knockout model (Amh<sup>cre/+;Smad4<sup>−/−</sup></sup> or Smad4 cKO) would result from the inability of Sertoli cells to respond to fetal Leydig cell-derived activin A.

A time-course analysis of testis cord loop development indicated that Smad4 cKO testes developed similarly to those of controls at E15.5 (Fig. 1C). However, the testis cords of E17.5 and E19.5 Smad4 cKO embryos displayed minimal coiling (Fig. 1F and I). The impairment of testis cord expansion in Smad4 cKO testes was grossly similar in appearance to the defects in Inhba cKO embryos. Sagittal sections of both Inhba cKO (Fig. 1K) and

Fig. 1. Testis cord development in Inhba cKO, Smad4 cKO, and control mouse embryos. (A–I) Developmental time course of testes from control (A, D, and G), Inhba cKO (B, E, and H), and Smad4 cKO (C, F, and I) embryos from E15.5 to E19.5. Transverse sections were stained with laminin (green) to demarcate boundaries of testis cords. White arrows indicate underdeveloped testis cords. (Scale bar, 100 μm.) (J–O) H&E-stained sagittal sections from E19.5 control (J and M), Inhba cKO (K and N), and Smad4 cKO (L and O) testes are shown at 4x (J–L, scale bar, 250 μm) and 40x (M–O; scale bar, 100 μm). Black arrows indicate Sertoli cells aberrantly located in the testis cord lumen. rt, Rete testis.

Control
Inhba cKO
Smad4 cKO

E15.5
A
B
C
D
E
F
G
H
I

E17.5
J
K
L
M
N
O

EM stain of sagittal testis sections at E19.5.
**Smad4 cKO (Fig. 1L) E19.5 testes revealed fewer testis cord cross-sections; in addition, the diameter of individual cross-sections was enlarged compared with controls (Fig. 1J). High-magnification images revealed that Sertoli cells sloughed off into the testis cord lumen in both Inhba cKO (Fig. 1N) and Smad4 cKO (Fig. 1O) testes. The similar morphological defects in Inhba cKO and Smad4 cKO testes indicate activin A from the fetal Leydig cells signals to Sertoli cells. These results also suggest that activin A is the primary TGF-β superfamily ligand acting upon the fetal Sertoli cell epithelium.**

**Testis Cord Dysgenesis in Leydig Cell-Specific Inhba and Sertoli Cell-Specific Smad4 Knockout Embryos Results from Decreased Sertoli Cell Proliferation.** Testis cord coiling during late fetal development coincides with, and is presumably driven by, the rapid proliferation of Sertoli cells (28). To determine whether fetal Leydig cell-derived activin A promotes Sertoli cell proliferation during this critical time, we analyzed testes of E19.5 control and cKO mice. Proliferating Sertoli cells were identified via immunohistochemistry for the proliferation marker Ki67 (Fig. 2). In control mice, numerous proliferating Sertoli cells outlined the testis cords at E19.5 (Fig. 2A). In both Inhba cKO (Fig. 2B) and Smad4 cKO (Fig. 2C) testes, proliferation within the Sertoli cell epithelium was dramatically reduced. Quantitative analysis revealed that the percentage of Ki67-positive Sertoli cells was significantly decreased in Inhba cKO (Fig. 2D) and Smad4 cKO (Fig. 2D) newborn testes compared with controls (P < 0.0001 versus control; n = 3 for Smad4 cKO and n = 7 for other genotypes). These data suggested that reduced Sertoli cell proliferation could be the cause of defective testis cord expansion in Inhba cKO and Smad4 cKO embryos. Sertoli cell proliferation was also significantly decreased in Smad4 cKO testes compared with Inhba cKO testes (P < 0.01) despite the histological similarities between these two models (Fig. 1K and L). It is possible that partial compensation by other TGF-β superfamily ligands could lead to a higher rate of Sertoli cell proliferation in Inhba cKO testes when compared with Smad4 cKO embryos. In Smad4 cKO testes, loss of Sertoli cell Smad4 would preclude a canonical response to any TGF-β superfamily proteins.

**Testicular Dysgenesis Persists into Adulthood in Leydig Cell-Specific Inhba and Sertoli Cell-Specific Smad4 Conditional Knockout Mice.** Although proliferation of fetal Sertoli cells does not rely upon pituitary-derived gonadotropins, postnatal proliferation is controlled by systemic hormones such as FSH (29). Although genetic alteration of testicular activin A signaling led to decreased Sertoli cell numbers by the time of birth, it is possible that this reduced population of Sertoli cells could proliferate at a normal or even increased rate postnatally as their proliferation becomes FSH dependent. We thus evaluated testis development and function in 8- to 16-wk-old adult Inhba cKO and Smad4 cKO males as well as their respective age-matched and genetic background-matched controls (Fig. 3). A reduction in testis size and weight was evident in both Inhba and Smad4 cKO males despite no significant differences in body weight among genotypes (Fig. 3 A–D and G). In addition, daily sperm production (DSP) per gram of testis weight was significantly lower in Inhba and Smad4 cKO males compared with control males (Fig. 3 E and F). This supported an underlying inefficiency of spermatogenesis in our models, as the testes of our models produced fewer sperm than controls even when differences in testis size were taken into account (P < 0.05). No significant differences were found among Inhba cKO, Smad4 cKO, and control mice with regard to androgen-sensitive endpoints such as anogenital distance and seminal vesicle weight, indicating that defects in spermatogenesis did not result from androgen insufficiency (Fig. 3G).

To further investigate the phenomenon of inefficient spermatogenesis, we analyzed testis histology and found that, compared with controls (Fig. 4A), seminiferous tubule diameters in Inhba cKO (Fig. 4B) and Smad4 cKO (Fig. 4C) adult males were generally enlarged. Testis cord diameter was also enlarged in Inhba and Smad4 cKO fetal testes (Fig. 1 N and O), suggesting a continuance of this fetal phenotype. The presence of numerous seminiferous tubule cross-sections in Inhba cKO and Smad4 cKO testes indicated that postnatal coiling did occur. In addition to partial recovery of seminiferous tubule coiling, grossly normal spermatogenesis was evident in both Inhba and Smad4 cKO testes (Fig. 4 B and C). However, examination of testis histology at higher magnification revealed focal dysgenic tubules with abnormal or even absent spermatogenesis (Fig. 4 G and H) scattered among functionally normal tubules in Inhba and Smad4 cKO testes.
characterization that has seemed only fair considering that fetal Leydig cells are not known to have roles outside of steroid production and the promotion of testis descent. In this study, we have uncovered a unique and active role of fetal Leydig cells during testis cord expansion. To our knowledge, activin A is the first fetal Leydig cell-produced factor shown to signal to Sertoli cells and regulate their proliferation during fetal testis development. Although Sertoli cell proliferation is dramatically decreased in embryos with disruption of either activin A or SMAD4, it is not entirely abolished, suggesting other unidentified signaling factors may compensate for the loss of fetal Leydig cell-derived activin A signaling. In addition, the design of our study does not rule out the possibility that other TGF-β superfamily ligands may influence Sertoli cell proliferation via noncanonical pathways (those not requiring SMAD4). The presence of low levels of Sertoli cell proliferation in Inhba cKO and Smad4 cKO testes may imply that factors coming from the Sertoli cells themselves also promote proliferation, particularly in the absence of activin A signaling. The higher levels of fetal Sertoli cell proliferation that we observed in Inhba cKO mice compared with Smad4 cKO mice could indicate compensatory effects of other TGF-β superfamily ligands during this process. This inequity of Sertoli cell proliferation may also have been influenced by the background strain differences of our models, as well as the difference in timing of cre recombinase activity due to the transgenic strains used (20, 22). Although we cannot confirm that activin A is the sole signaling factor involved in fetal Sertoli cell proliferation, our findings bring to light the major contribution of fetal Leydig cell-derived activin A to Sertoli cell proliferation and thus testis cord expansion.

**Activin A Is Involved in Mesenchymal–Epithelial Cross-Talk Required for Testis Cord Expansion.** Although many communications from epithelium to mesenchyme have been elucidated during fetal testis morphogenesis, activin A is one of the few factors shown to signal from the mesenchyme to the epithelium. This mesenchymal–epithelial cross-talk is better understood in the adult testis, where adult Leydig cells produce signals critical for the maintenance of Sertoli cell function and, ultimately, sperm production. For example, the actions of adult Leydig cell-derived testosterone upon Sertoli cells are critical for the normal progression of spermatogenesis (30). However, this cross-talk does not exist in the embryo, as fetal Sertoli cells do not express androgen receptor (31). Our discovery of the actions of mesenchymal activin A on epithelial development raises the possibility that other unidentified mesenchyme-derived factors could play essential roles in testis cord development.

In addition to identifying activin A as a product of the fetal Leydig cells, we have uncovered an unusual role for this signaling protein during tubulogenesis. In many tubular organs, loss of activin A function results in ectopic epithelial branching; therefore, activin A is thought to function primarily as an inhibitor of branching morphogenesis (13, 17, 15). This role is not dependent upon the compartmental expression pattern of activin A. In the embryonic kidneys and lungs, activin A is produced by the epithelium, whereas pancreatic activin A is expressed in both epithelium and mesenchyme (13, 17, 32, 33). However, disruption of activin A signaling in these tissues leads to ectopic branching of the tubular structures. Within the context of the embryonic testis, activin A serves as a stimulatory factor driving epithelial proliferation. We did not observe ectopic epithelial branching in the testis cords of our murine models lacking testicular activin A signaling. The role of activin A in the fetal testis is actually most similar to its functions in the teeth and epididymes. In the developing teeth, mesenchymal activin A promotes epithelial cell proliferation that allows for the progression of incisors and mandibular molars beyond the tooth bud stage (14, 34). Similarly, mesenchymal activin A in the developing Wolffian duct

<table>
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<th>Genotype</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Anogenital distance (mm)</th>
<th>Seminal vesicle weight (mg)</th>
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<tr>
<td>Inhba control</td>
<td>4</td>
<td>26.42 ± 0.88</td>
<td>15.03 ± 0.39</td>
<td>206.06 ± 27.34</td>
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<tr>
<td>Inhba cKO</td>
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<td>14.43 ± 0.80</td>
<td>211.98 ± 23.03</td>
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<tr>
<td>Smad4 control</td>
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<td>27.93 ± 2.07</td>
<td>15.27 ± 0.75</td>
<td>356.96 ± 62.64</td>
</tr>
<tr>
<td>Smad4 cKO</td>
<td>7</td>
<td>30.82 ± 6.37</td>
<td>15.60 ± 1.16</td>
<td>355.37 ± 95.95</td>
</tr>
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Fig. 3. Testis development and sperm production in 8- to 16-wk-old Inhba cKO, Smad4 cKO, and control mice. (A and B) Images of representative Inhba cKO and cKO testes (A) as well as Smad4 cKO and cKO testes (B). (C and D) Daily sperm production (DSP). *Statistical difference of $P < 0.05$ (n = 4 for Inhba control, n = 5 for Inhba cKO, n = 10 for Smad4 control, and n = 7 for Smad4 cKO). (G) Average body weight, anogenital distance, and seminal vesicle weight. Values are stated as mean ± SD. For each parameter, Inhba cKO, and Smad4 cKO mice do not statistically differ from their strain-specific controls ($P > 0.05$).
but also inhibin A, a receptor-level antagonist of activin signaling — a pathway that has been shown to be important in the regulation of testis development (36). In our two models are evident from embryonic development through adulthood. In both models, alteration of testicular activin A signaling during fetal life manifested as reduced testis size, lower sperm production, and abnormal testicular histology in adulthood.

The similarities between the two murine models were somewhat surprising, given the complexities of the TGF-β superfamily in male reproduction. By removing Inhba in the fetal Leydig cells, we precluded these cells from producing not only activin A but also inhibin A, a receptor-level antagonist of activin signaling (37). However, there is convincing circumstantial evidence to indicate that the testicular dysgenesis we observed in our Inhba cKO mice resulted from loss of activin A rather than inhibin A. First, the phenotype of inhibin α (Inha) null mice, which can still produce activins but which lack the ability to produce inhibins, differs significantly from our murine models. Fetal testis cord dysgenesis has not been reported in Inha-null mice; however, these mice develop gonadal tumors, a phenotype not observed in our murine models even in advanced age (38). Second, during spermatogenesis, it is Sertoli cell-derived inhibin B, rather than inhibin A, that functions as the major gonadal inhibin providing feedback to the hypothalamus (39). The presence of grossly normal spermatogenesis in our Inhba CKO mouse model suggests that removal of Inhba from the fetal Leydig cells does not alter the ability of the Sertoli cells to produce inhibin B and thus provide hypothalamic feedback. Taken together, these observations support loss of activin A, not inhibin A, as the causal factor behind testicular dysgenesis in Inhba CKO and Smad4 cKO mice.

In a larger context, our findings provide models supporting the potential fetal origins of adult testicular dysgenesis (40). Interestingly, studies of activin pathway components in human fetal testes mirror the pattern observed in mice; specifically, activin A localizes to interstitial fetal Leydig cells, whereas the Sertoli cell epithelium expresses activin receptors (41). Thus, activin A regulation of testis cord expansion may be conserved in humans and mice. In humans, an increasing body of epidemiological evidence indicates that semen quality is diminishing in many developed countries; however, the precise reasons for these trends are unclear (40, 42). Further study of murine models related to the testicular activin A pathway may provide valuable insight into the developmental origins of human reproductive disorders such as idiopathic infertility and androgen-independent spermatogenic failure.

**Materials and Methods**

**Generation of Conditional Knockout Mice.** To produce Inhba cKO mice (Amhr2cre;Inhbafl/fl), Inhba<sup>−/−</sup> animals were mated to Amhr2<sup>cre/cre</sup> transgenic mice (20, 43). The resulting Amhr2<sup>cre/cre</sup>;Inhba<sup>−/−</sup> mice were then crossed to Inhba<sup>fl/fl</sup> animals (21). Smad4 cKO (Amhr2<sup>cre/cre</sup>;Smad4<sup>fl/fl</sup>) mice were generated.

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**Fig. 4.** Spermatogenesis in 8- to 16-wk-old Inhba cKO, Smad4 cKO, and control mice. (A–C) H&E-stained histological sections at 10x magnification. (Scale bar, 250 μm.) (D–H) Higher magnification (20x) reveals areas of normal spermatogenesis in control (D), Inhba cKO (E), and Smad4 cKO (F) testes as well as focal areas of aberrant spermatogenesis in Inhba cKO (G) and Smad4 cKO (H) testes. Asterisks indicate dysgenic tubules. (Scale bar, 100 μm.)
by mating Smad4−/− mice to Amhr2cre/+ transgenic mice; the resulting Amhr2cre+/−; Smad4−/− mice were then crossed to Amhr2cre+/− animals (22, 23). Timed matings were performed by housing female mice with males overnight and checking for vaginal plugs the next morning (E0.5 = noon of the day when a vaginal plug was found). Fetal tissue was collected from E12.5 to E19.5. For checking for vaginal plugs the next morning (E0.5 = noon of the day when litters were produced by housing female mice with males overnight and adult analysis, by mating females were collected at 8–18 wk of age. All procedures described were reviewed and approved by the Institutional Animal Care and Use Committee, and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD), DHHS Publ No (NIH) 85-23.

Immunohistochemistry and Histology. For immunohistochemistry, tissues were fixed in 4% paraformaldehyde, dehydrated through a sucrose gradient, and cryosectioned. Primary antibodies included those specific to laminin and Ki67. Ki67 immunohistochemistry was performed on E19.5 frozen sections following instructions included with the TSA Fluorescein System Kit (Perkin-Elmer). A minimum of three animals per genotype were analyzed. Per animal, at least four alternating tests cross-sections a minimum of 40 μm apart were photographed. The percentage of proliferation was calculated as the number of Ki67-positive Sertoli cells divided by the total number of Sertoli cell nuclei. Because values did not statistically differ among control genotypes, only data from Inhba control testes are shown. Sections were counterstained with mounting media containing DAPI. For histological analysis, E19.5 and adult samples were fixed in Bouin’s solution, and paraffin sections were stained with H&E.

Daily Sperm Production. Analysis and calculation of daily sperm production followed the procedure of Joyce et al. (44) with slight modifications. Testes were homogenized for 30 s using a Polytron homogenizer, and sperm heads were then counted on a hemacytometer.

Statistical Analysis. Statistical differences were determined via two-tailed t test comparisons.

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