BOR Papers in Press. Published on May 30, 2012 as DOI:10.1095/biolreprod.111.097527



**Title:** Overexpression of Follistatin in the Mouse Epididymis Disrupts Fluid Resorption and Sperm Transit in Testicular Excurrent Ducts.
**Short Title:** Elevated follistatin causes epididymal pathology.

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This research was supported by the National Institutes of Health (R01-CA090398) to R.A.K.

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**ABSTRACT**
Activin is a well established modulator of male and female reproduction that stimulates the synthesis and secretion of follicle-stimulating hormone. Non-pituitary effects of activin have also been reported, although the paracrine actions of this growth factor in several reproductive tissues are not well understood. To identify the paracrine functions of activin during mammary gland morphogenesis and tumor progression, we produced transgenic mice that overexpress follistatin (FST), an intrinsic inhibitor of activin, under control of the mouse mammary tumor virus (MMTV) promoter. Although the MMTV-*Fst* mice were constructed to assess the role of activin in females, expression of the transgene was also observed in the testes and epididymides of males. While all 17 transgenic founder males exhibited copulatory behavior and produced vaginal plugs in females, only one produced live offspring. In contrast, transgenic females were fertile, permitting expansion of transgenic mouse lines. Light and transmission electron microscopic examination of the transgenic testes and epididymides revealed impairment of fluid resorption and sperm transit in the efferent ducts and initial segment of the epididymis, as indicated by accumulation of fluid and sperm stasis. Consequently, a variety of degenerative lesions were observed in the seminiferous epithelium, such as vacuolation and early stages of mineralization and fibrosis. Sperm collected from the caudae epididymidis of MMTV-*Fst* males had detached heads and were immotile. Together, these data reveal that activin signaling is essential for normal testicular excurrent duct function and that its blockade impairs fertility. These results also suggest that selective inhibitors of activin signaling may provide a useful approach for the development of male contraceptives without compromising androgen synthesis and actions.

**Summary Sentence:** Follistatin overexpression in the testis and epididymis of transgenic mice causes infertility due to defects in fluid resorption, sperm transit and morphology in excurrent ducts.

**Key Words: follistatin, epididymis, testis, fertility, mouse**

## INTRODUCTION

Follistatin (FST) and activin were first isolated in the 1980s as peptides found in ovarian follicular fluids that could either inhibit or activate the production and secretion of follicle-stimulating hormone (FSH) from the pituitary gland, respectively [1, 2]. While activin could feed back at the pituitary gland to activate synthesis and release of FSH, FST was found to modulate FSH release by directly binding to, and inhibiting the activity of, activin. This duo of proteins was thought to function as an endocrine regulatory loop between the gonads and pituitary gland to modulate reproductive potential. Over the last 30 years, identification of FST and activin subunits in a wide variety of both reproductive and non-reproductive tissues suggests that in addition to regulating FSH, these factors contribute to a plethora of physiological functions including branching morphogenesis of many organs including the kidney, pancreas and salivary glands, wound repair, inflammation response, and bone remodeling, among others, by acting in both autocrine and paracrine fashions [3-7].

Activins are members of the TGF-β superfamily of ligands that exist as β-subunit dimers, unlike their sister family members, the inhibins, which consist of heterodimers of an α-subunit and one of five β-subunits (βA- β-E). Activin A is a homodimer of two βA subunits, while Activin AB is a heterodimer of one βA and one βB subunit. Secreted activin binds to either the Activin Type IIA (ACVR2a) or Type IIB (ACVR2b) receptors, both of which are constitutively active. This leads to recruitment and transphosphorylation of ActRIB receptor (ALK4), which in turn phosphorylates the signaling intermediates, Smads 2 and 3. The phosphorylated receptor-regulated Smads then bind the common mediator, Smad4 and the complex translocates to the nucleus where it binds DNA and modulates transcription of target genes.

FST, a monomeric and highly glycosylated polypeptide, is an endogenous inhibitor of activin. Two secreted FST molecules irreversibly bind an activin subunit dimer with high affinity, thereby blocking the ability of activin to interact with its cognate receptors and activate downstream signaling [8]. FST and activin are produced in many of the same tissues, suggesting that FST provides a finely tuned mechanism for regulating activin activity. While FST binds to activin with high affinity (~50-450 pM), it has also been shown to bind and inhibit other members of the TGF-β superfamily in mammals including Bone Morphogenetic Proteins (BMPs) 4, 5, 6, 7, 11, 15 and GDF-9, as well as myostatin, albeit with much lower affinity (0.5-40 nM) [8-15]. FST binds and inhibits the activity of BMP2 in *Xenopus* as well [10, 16]. There are three isoforms of FST which vary in their cellular localization [17]. Two isoforms are splice variants produced from the *Fst* gene. The FST315 isoform contains all 6 exons and is primarily found in the systemic circulation. FST288 is the smallest isoform, lacking exon 6, thereby losing the C-terminal tail. This truncated isoform which displays a higher affinity for heparin sulfate proteoglycans on the cell surface, anchoring FST to the cell membrane. The third isoform, FST303, is produced by post-translational proteolytic cleavage and has a shorter C-terminal tail than FST315. This isoform can bind to the cell surface but with lower affinity than FST288. While all three isoforms bind activin with comparable affinities [17], they vary somewhat in tissue distribution.

The in vivo study of the roles of activin and FST in male reproduction has been hampered due to the essential role of each of these molecules in fetal development. Mice with deletion of the activin-βA subunit, ActRIIB (*Acvr2b*), ActRIA (*Alk2*), ActRIB (*Alk4*) or FST die any time from early gastrulation to shortly after birth [18-21]. Disruption of ActRIIA (*Actr2a*) in mice also causes ~22% embryonic mortality; however, male mice that survive are fertile albeit with delayed sexual maturation [18]. In contrast, males with disruption of the activin-βB subunit are viable and fertile suggesting that activin A has a more profound biological impact than activin-βB. Insertion of activin-βB into the locus of the activin- βA partially rescued neonatal lethality and craniofacial defects of the activin- βA null mice. However, the seminiferous tubule differentiation was delayed in males, testicular volumes were reduced and fertility onset delayed [22]. These data indicate that male reproductive function specifically requires activin-βA. Furthermore, mice with targeted deletion of activin-βA in fetal Leydig cells disrupts testis cord elongation and expansion, culminating in low sperm production and testicular pathology in adulthood [23].

Transgenic mice have also shed light on the role of activin and FST in male reproduction. Overexpression of the Activin βC subunit in Sertoli cells blocks Activin A activity and causes male infertility [24]. Overexpression of FST, or its related protein Follistatin-like 3 (FSTL3), were expected to phenocopy the surviving ActRIIA-deficient male mice. However, adult FST transgenic males display decreased fertility and reduced testicular weight [25]. Similarly, transgenic mice that overexpress FSTL3 under control of the *Inha* promoter in Sertoli cells are subfertile, with decreased testicular size [26]. These studies confirm that activin signaling in the testes is essential for normal function.

While transgenic mice have been helpful in elucidating the reproductive ramifications of altered activins and FST in the testes, the roles that these two molecules play in excurrent ducts (efferent ducts and epididymis) remain unknown. The epididymis is a highly convoluted duct that connects the rete testis and efferent ducts to the vas deferens and provides a complex environment for the maturation and transport of sperm. The epididymis, along with the efferent ducts, resorbs testicular fluid, differentially endocytosing some luminal fluid-borne proteins while secreting new ones [27-29], thus imparting on sperm the ability to move and fertilize an egg. This sperm maturation [30] occurs before sperm reach the cauda epididymidis where they are stored until ejaculation. Nearly 90% of this resorption occurs in the efferent ducts and, on a per sperm basis, the amount of protein present in the central caput epididymidis is less than 15% of that leaving the testis. The resorption of fluid and differential endocytosis in the efferent ducts and along the length of the epididymis are essential for fertility as perturbation of these processes leads to retention of fluid, elevated pressure in the testis and impaired spermatogenesis [30-32]. Endogenous expression of FST and activin β-subunits have been documented in the epididymis in humans, primates, pigs and mice, and in some cases, is found at levels higher than in the testes [33-35]. While little is known regarding activin receptor localization in the adult epididymis, ActRIIB and phosphorylated Smads 2/3 are present in the Wolffian epithelium suggesting that activin signaling is operational during epididymal development [36]. Indeed, activin-βA subunit is essential for mouse epididymal coiling, substantiating the importance of activin in excurrent ducts [37].

Herein, we describe a novel murine model that overexpresses follistatin in the testes and epididymides using the Mouse Mammary Tumor Virus (MMTV) promoter [38]. Breeding attempts with male founder mice revealed a dramatic infertility phenotype. Characterization of this phenotype unveils an essential role for FST/activin homeostasis in maintaining excurrent ductal function and reproductive performance.

## MATERIALS AND METHODS

*Generation of MMTV-Follistatin Transgenic Mice*

The MMTV-LTR promoter was obtained from Kay-Uwe Wagner [38] and was cloned with HindIII into pBluescript SK+ containing the bovine Growth Hormone Intron/Poly A (bGHpolyA). The mouse *Fst* gene, from Martin Matzuk [25], was digested with XbaI, the ends blunted with Klenow and ligated into the MMTV-bGHpolyA-pBluescript vector with EcoRV. The signal peptide of FST located in exon 1 is intact in this construct; hence an increase in FST secretion is expected from all cells capable of activating the MMTV promoter. The MMTV-*Fst* transgene was microinjected into fertilized oocytes by the Case Transgenic and Targeting Facility. Mice were genotyped by PCR with primers specific to the *Fst* gene (FORWARD 5'-CTTAACGCAGTTGCCACCTA-3)' and the 3' end of the *Fst* gene overlapping with the bGHpolyA tail (REVERSE 5'-AAGCAGGAGAGCAGACCGTA-3'). Once MMTV-*Fst* transgenic males were determined to be infertile, female founders were bred to FVB/N males (Jackson Laboratories, Bar Harbor, ME, USA) to expand lines. The original male transgenic founders as well as offspring from three independent transgenic lines generated from female founders (designated as Lines 'A', 'B' and 'C') were used to study the extent of the infertility in MMTV-*Fst* males. Age-matched, non-transgenic male littermates were used as wild type (WT) controls for all experiments. Mice were housed in microisolator cages under pathogen-free conditions with a 12-h light/dark cycle and provided food and water ad libitum. All animal studies were approved by the Case Western Reserve University or Colorado State University Institutional Animal Care and Use Committees.

*Fertility Evaluation*

At 8.5 weeks of age, 17 MMTV-*Fst* founder males as well as 5 wild type (WT) males were individually housed in cages with one FVB/N female and monitored for copulatory behavior, presence of vaginal plugs, and litters for 4 weeks, during which time all WT males produced offspring. FVB/N females were then randomly collected from the breeding cages of the 5 MMTV-*Fst* males as well as the 5 WT males and the females were monitored for approximately 4 weeks to ensure that they were not pregnant. The 5 females that conceived in the WT breedings, now considered proven female breeders, were placed in the cages with MMTV-*Fst* transgenic founders and monitored for 4 additional weeks for the presence of litters. The original females from the MMTV-*Fst* matings were housed with the WT males and also monitored for the generation of litters.

For superovulation studies, 3-week-old FVB/N females (Jackson Laboratories, Bar Harbor ME, USA) were superovulated using a previously described PMSG (pregnant mare serum gonadotropin, Calbiochem, La Jolla, CA) and hCG (human chorionic gonadotropin, Wyeth-Ayerst Laboratories Inc., Philadelphia, PA) regimen [39]. Five MMTV-*Fst* and 3 WT males were each housed with one superovulated female on two separate occasions and breedings were monitored until pups were born.

*Serum Hormone Analyses*

Whole blood was collected from the retro-orbital sinus of 6-10 month old mice under avertin anesthesia as well as from cardiac puncture at the end of the study. Blood was clotted overnight at 4°C, centrifuged and serum collected. Serum was assayed for luteinizing hormone (LH) by sandwich immunoradiometric assay (IRMA), and testosterone (T) and FSH by radioimmunoassay (RIA) at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

*Organ Weights and Histology*

Mice (aged 6-10 months) were weighed, killed by $CO_2$ asphyxiation and blood collected by cardiac puncture. Testes and seminal vesicles were dissected and weighed. Testis and epididymis from one side were fixed in 4% paraformaldehyde for 4 hours and transferred to PBS. Fixed tissue was embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). For FST immunostaining (IHC), sections were deparaffinized, rehydrated and antigen retrieval was performed in 10 mM sodium citrate buffer, pH 6.0 for 20 minutes using a decloaking chamber (Biocare Medical). After quenching endogenous peroxidases and blocking in TBS-Tween-20 plus BSA, the primary antibody, goat α-FST [R&D Systems (AF669)] or IgG as a negative control, was applied at 2 μg/ml overnight at 4°C. Bound antibody was detected the following morning with the Vector Elite ABC kit (Vector Laboratories) using 3, 3'-diaminobenzidine (DAB). Immunostaining for the estrogen receptor [ER, Santa Cruz (MC-20)] was performed as previously described [40]. Sections were counterstained with Gills #3 hematoxylin (Fisher), dehydrated and mounted in Permount (Fisher). Goat or rabbit IgG (Jackson ImmunoResearch Inc.) was substituted for the primary antibody as a negative control. Images were captured using a Nikon microscope equipped with Zeiss AxioCam HRc imaging system.

*RNA Analysis*

RNA was isolated from brain, heart, liver, lung, kidney, testes, epididymides and efferent ducts of 6-10 month old mice using Trizol Reagent (Invitrogen) and DNase-treated (DNA-Free, Ambion). cDNA was generated using Superscript II and random hexamers (Invitrogen) as per the manufacturer's protocol. Quantitative Real-Time PCR (qPCR) was performed on an Applied Biosystems Step One Plus Real-Time PCR system using TaqMan Gene Expression Assays for mouse *Fst* (Mm00514982_m1) and *Esr1* (Mm01191130_m1) and normalized to *Gapdh* (4352932-0804021 ).

*Sperm Evaluation*

The left testis and epididymis were removed from anesthetized (ketamine/xylazine) 7-month-old males after examination of visceral organs. Three FST-expressing and 2 WT littermates from line A and 2 FST-expressing and 1 WT littermate males from line B were used. The epididymis was dissected from the left testis, individual organs were weighed. The epididymis was placed in 1.0 ml of PBS (300 mOsm; prepared with endotoxin free $H_2O$) and the cauda was fenestrated with a 25 ga needle. After a 5 min incubation at 37°C, 5% $CO_2$, a 50 μl epididymal fluid sample was used to assess total motility using a video microscope. An additional 50 μl epididymal fluid sample was fixed in 0.25 ml of 10% buffered formal saline to assess sperm morphology (500X magnification), and sperm counts using a hemocytometer (125X magnification) with a phase contrast microscope. When available, 200 sperm, from each male were classified as morphologically normal or abnormal, with abnormalities categorized as abnormal head shape, the presence of a retained cytoplasmic droplet, reflected midpiece, and detached (tail-less) heads [41]

*Transmission Electron Microscopy*

After removal of the left testis and epididymis for light microscopic evaluation, the anesthetized animals were perfused with 2% glutaraldehyde and the right testis and epididymis were collected. Four samples of tissue, two from the testes representing the proximal pole adjacent to the efferent ducts and middle portions, one from efferent ducts/initial segment of the epididymis, and one from cauda epididymidis, were post-fixed in 1% osmium tetroxide in cacodylate buffer (0.1M) and embedded in Poly/Bed 812 (Polysciences, Warrington, PA, USA). A series of one-µm-thick sections of testes and epididymides were cut (n=~30-50 sections/animal), stained with toluidine blue, and evaluated using a Nikon Microphot FXA microscope (equipped with plan apochromatic, bright-field/differential interference contrast optics) interfaced with a computerized imaging system. Images were captured using ImagePro software (Media Cybernetics, Inc.). Thin sections (60–80 nm) were cut from Poly/Bed-embedded tissues and stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (JEOL-1200EX, JEOL USA, Inc., Peabody, Massachusetts).

*Statistical Analyses*

The data are expressed as means ± standard deviations. Statistical analyses were performed using a one-way analysis of variance, followed by Student's *t* -test. A minimum p value of <0.05 was considered statistically significant.

## RESULTS

*Male MMTV-*Fst *transgenic founders are infertile*

Thirty-two transgenic founder mice (17 males and 15 females) were generated with the wild type mouse *Fst* gene driven by the MMTV-LTR promoter. All transgenic mice were physically indistinguishable from WT littermates, with similar body sizes and normal hair and tooth development. MMTV-*Fst* males were initially housed with one FVB/N female to expand lines, but no litters were produced (see breeding schemes in Figure 1). In contrast, MMTV-*Fst* founder females were mated with WT FVB/N males and uneventfully produced litters. Hence, transgenic females were used to expand several lines of transgenic mice for additional study. No further fertility studies were performed with the females. A more detailed assessment of the mammary gland phenotypes of MMTV-*Fst* females is in progress. To further investigate the conspicuous infertility of MMTV-*Fst* males, all 17 transgenic founder males were mated with WT FVB/N females. As a positive control, 5 of the WT founder males were also bred to WT FVB/N females. While all 5 of the WT founder males produced at least one litter of pups, only 1/17 (6%) transgenic founder males yielded any litters, despite the observation of vaginal plugs and normal male copulatory behavior (Table 1). To exclude the possibility that the WT females used for this study were infertile, we replaced 5 of the females housed with the MMTV-*Fst* founder males with the 5 proven females that had successfully generated litters when mated with WT males. In addition, 5 females from the MMTV-*Fst* breedings were placed with WT males. Again, all 5 of the WT males produced litters while 0/5 of the MMTV-*Fst* males sired offspring (Table 1). To further confirm that the MMTV-*Fst* male mice were infertile, 5 MMTV-*Fst* males and 3 WT males were mated with superovulated females. Again, no litters resulted from

MMTV-*Fst* males while all 3 of the WT males generated litters (Table 1). This superovulation experiment was repeated once with the same outcome. From these data, we conclude that the *Fst* gene driven by the MMTV-LTR promoter results in a defect that renders male mice infertile. Male offspring of the female founder breedings were also confirmed to be infertile, indicating that the infertility phenotype was heritable and restricted to males (Figure 1B).

*Infertility in MMTV-FST males is not due to systemic disruption of pituitary gonadotropins or testosterone*

Activin plays an essential role in regulating the synthesis and release of FSH from the pituitary gland and subsequent steroidogenesis. Serum testosterone and FSH were quantified in adult MMTV-*Fst* founders or F1 progeny from female founders (lines FST-A and FST-B) and their WT littermates (Figure 2A). While some of the MMTV-*Fst* males exhibited elevated levels of testosterone, many of the founders had levels within the normal range but were infertile. Similarly, circulating FSH levels in infertile F1 males were only modestly elevated and most males were within the normal range (Figure 2B). Moreover, while LH levels were elevated to a greater extent in some MMTV-FST mice, several infertile males also maintained normal levels (Figure 2C). The changes in serum gonadotropins or testosterone were not consistently altered in infertile males, indicating that endocrine dysfunction was not the primary cause of infertility.

*Infertility is not consistently associated with a decrease in testis weight*

Previous studies have shown that disruption of activin signaling via ligand manipulation or enhanced FST or FSTL3 expression within the testes impacts testes weights [24-26]. The testes and seminal vesicles in adult WT and MMTV-*Fst* transgenic mice were weighed and, consistent with serum hormone levels, organ weights were variable both within and between infertile transgenic lines (Figure 2D-E). No differences in body weights were observed between WT and transgenic mice in any line examined (data not shown). Testes weights were decreased by as much as 80% in Line B, not changed in Line A, and were intermediate in Line C (decreased 17%), even though all mice examined from these lines were infertile. It is expected that a mere accumulation of testicular fluid might initially increase the organ weight, whereas the degenerative changes in the seminiferous epithelium (which lead to fibrosis over time) consequent to impediment of fluid resorption and transit might ultimately decrease the weight. Interestingly, while reports have shown inverse correlations between testicular FST transgene expression and testicular weights [25, 26], this did not occur with targeted expression of FST driven by the MMTV promoter (Figure 3A). This may simply be a matter of the time at which the parameter is measured relative to the timeline of the progression of testicular lesions.

Male fertility is directly linked to Sertoli cell number which is established during postnatal development [42]. Together with FSH, activin stimulates the initial wave of Sertoli cell proliferation between postnatal days 4-9 in rodent testes [43-46]. To determine if FST overexpression in the testes could abrogate the FSH/activin-induced initial wave of Sertoli cell proliferation and lead to a smaller testicular weight, we harvested testes from post-natal days 5 and 8 of WT and MMTV-*Fst* pups and evaluated transgene expression and relative testicular size. FST protein was below the level of detection by IHC in the postnatal testes of transgenic mice and the size of the transgenic testes was indistinguishable from WT mice at both days examined (data not shown). This indicates that the transgene is inactive at these days and that early suppression of Sertoli cell number is unlikely to be the underlying cause of the reduced testicular weights or the infertility of adult MMTV-*Fst* male mice. Indeed, smaller testicular size and reduced sperm counts have been documented in FSH-β and ACTRIIA null mice, while both mouse models remain fertile [18, 47]. Seminal vesicle weights were also compared between adult WT and MMTV-*Fst* mice and surprisingly are only modestly elevated in one of the three transgenic lines examined (Line B, +30%) despite an overall elevation in serum testosterone levels (Figure 2E). As observed for testes weight, serum gonadotropin levels and testosterone levels, seminal vesicle weights for many infertile mice were similar to those of WT mice.

*FST is overexpressed in the testes, efferent ducts epididymides of juvenile and adult transgenic mice*

We next evaluated the level and localization of transgene activity in the male reproductive tract. F1 MMTV-*Fst* transgenic males had elevated levels of *Fst* mRNA in the testes compared to WT male littermates (Figure 3A). *Fst* mRNA was also increased in the epididymides (Figure 3B) and in the efferent ducts (Supplemental Figure S1, all Supplemental Data are available online at www.biolreprod.org). Immunohistochemical staining revealed that FST protein was similarly elevated in both the testes and epididymides (Figure 3C). Specifically in the testes, FST overexpression was localized to the Leydig cells, in agreement with previous reports of MMTV-LTR promoter activity [48]. In the epididymides, the MMTV promoter targeted overexpression to the principal epithelial cells. In addition, we observed a range of FST expression in other organs of the adult male transgenics from Line FST C, including brain, heart, liver, lung, and kidney (Supplemental Figure S2). Given that the FST transgene was not active in neonatal testes (data not shown), we determined if FST was overexpressed in the testes and epididymides of juvenile males at postnatal day 20 and day 24 . *Fst* mRNA was elevated at both time points in the testes, efferent ducts and juvenile epididymides of transgenic mice (Supplemental Figure S3), suggesting that FST overexpression during postnatal development may precipitate a cascade of aberrations culminating in the infertility phenotype observed in adults.

*FST overexpression causes fluid and sperm accumulation in the testes*

To further characterize the effects of elevated FST in the testes and epididymides, we examined the histological features of both organs. H&E-stained sections of WT testes exhibited normal spermatogenesis in the seminiferous epithelium except for focal vacuolation, which is not uncommon even in a healthy tubule (Figure 4A). In contrast, while normal spermatogenesis was evident in some seminiferous tubules of MMTV-*Fst* transgenic mice (Figure 4B), a spectrum of testicular lesions were evident. These included extensive germinal cell death and desquamation, seminiferous epithelial vacuolation and interstitial edema causing large spaces between the seminiferous tubules (Figure 4C and D). Other tubules with evidence of degeneration manifested sperm stasis and mineralization (Figure 4E-F). Engorged (fluid-filled) rete tubules, some containing denuded cells, were evident (Figure 4E). Together, these observations indicate that overexpression of FST induces an accumulation of fluid that leads to the pressure build-up and consequent degeneration of seminiferous epithelium. Importantly, FST overexpression does not appear to disrupt spermatogenesis as a result of a global hormonal defect because all stages of spermatogenesis were observed in some regions of the testes.

*Epididymal sperm transit and fluid reabsorption are impaired in MMTV-*Fst *transgenic mice*
A primary function of the rete testis, efferent ducts, and the initial segment of the epididymis is resorption and transport of luminal fluids exiting the testes. We postulated that the excessive fluid accumulation in the testes of MMTV-*Fst* males was the result of aberrant function of either fluid resorption or contractility in proximal excurrent ducts, predominantly the efferent ducts. Hence, we examined H&E-stained sections of the excurrent ducts of MMTV-*Fst* and WT litter-mate mice by light microscopy. In WT mice, there was no evidence of fluid accumulation or sperm stasis in the efferent ducts (Figure 5A). In addition, WT mice had normal spermiation and sperm transit as reflected by the normal luminal contents of the cauda epididymidis (Figure 5C). In contrast, accumulation of sperm in the efferent ducts was evident in MMTV-*Fst* mice (Figure 5B), indicating impaired fluid resorption and transit or contractile function. Reflecting the degenerative changes in the testes of MMTV-*Fst* mice, degenerate prematurely shed germ cells were evident in the lumina of the cauda epididymidis (Figure 5D) and distal corpus/proximal cauda (Figures 5E, F). The scarcity of tail-intact sperm was apparent in these epididymal regions of MMTV-*Fst* mice (Figures 5D-F). There was also an outpouching, or cystic formation, in the epithelial lining of the efferent ducts (arrow in Figure 5B) in one of the transgenic mice examined. Collectively, this histopathological profile indicates that overexpression of FST leads to impaired fluid resorption and/or contractility of the efferent ducts and initial epididymal segment, resulting in fluid accumulation, cystic outpouchings in excurrent ducts, and seminiferous epithelial degeneration.

*FST overexpression causes detachment of sperm heads and nuclear defects*
Modulation of activin ligand availability, receptors or alteration of FST within the testes leads to defects in spermatogenesis, sperm numbers and sperm motility [24-26]. As indicated above, normal spermatogenesis was evident in some seminiferous tubules of MMTV-*Fst* transgenic mice (Figure 4B). This indicates that spermatogenesis can proceed despite the locally elevated FST in the Leydig cells and that FST inhibition of activin or BMP ligands does not directly impact spermatogenesis. However, H&E sections of the caudae epididymidis revealed marked sperm degeneration (Figures 5D-F). To characterize the extent of sperm abnormalities, sperm was harvested from the caudae epididymidis of WT and transgenic mice and examined for motility and morphology. Sperm were present in all but one MMTV-*Fst* male that had only a single, intact, but immotile sperm in the sample examined. Less than 1% of the sperm from the MMTV-*Fst* males were motile, whereas the WT males demonstrated normal (45-75%) motility. The percentage of morphologically normal sperm were also significantly lower in the MMTV-*Fst* samples as compared to WT samples (3.4% vs. 24.5%, respectively); the predominant abnormality being a significant increase in the incidence of detached heads (Figure 6A, B). Due to the high frequency of detached (tail-less) heads, it was not possible to determine the incidence of retained cytoplasmic droplets and reflected midpieces in MMTV-*Fst* males as compared to WT males. In addition, transmission electron microscopy of cauda epididymal sperm revealed that a few sperm nuclei in MMTV-*Fst* mice had impaired chromatin condensation and nuclear craters (Figure 6C). While it is possible that FST has a role in spermiogenesis, this defect in sperm chromatin condensation along with the majority of sperm defects (i.e., detachment of heads and tails) observed in the FST transgenic mice are consistent with blockade of efferent ducts and consequent degeneration of seminiferous epithelium.

*Epididymal fluid resorption and sperm transit are impeded in FST-overexpressing mice*
Proximal excurrent ducts (efferent ducts and initial segment of the epididymis) were examined using transmission electron microscopy (Figure 7). In efferent ducts of WT mice, both ciliated as well as non-ciliated cells had normal apical specializations: junctional complexes between both cell types, cilia in ciliated cells, microvilli and elaborate endocytotic apparatus with coated pits, and tubulovesicular system in the non-ciliated cells (Figure 7A, B). In addition, the lumen was clear with presence of an occasional sperm profile as sperm transit in this region is normally very swift. Tight junctions between epithelial cells were indicative of epithelial integrity. In contrast, sperm had accumulated in the lumina of efferent ducts of MMTV-*Fst* transgenic mice (Figure 7D, E). The pressure resulting from the accumulated luminal contents caused the microvilli to be compressed and flattened. The compacted epithelial cytoplasm provided further evidence of increased ductal pressure. In addition, active phagocytosis of cellular debris (presumably fragmented sperm nuclei) was evident in the form of phagosomes. Endocytotic pits and vesicles were loaded indicating that the transcytosis pathway was saturated with material. On the basal aspect of the transgenic efferent ducts (Figure 7F), the lamina lucida of the basal lamina (also called lamina rara; the zone closest to the epithelium that contains laminin, heparan sulfate and cell surface integrins), was abnormally thickened with inclusions compared to the same component in the WT mice (Figure 7C). Likewise, the lamina densa underlying the lamina lucida was also thickened indicating ineffective basal transcytosis and fluid uptake by the lymphatics and blood vessels. Together these observations demonstrate that overexpression of FST in the efferent ducts causes sperm stasis due to impaired fluid resorption and/or contractility.

*Estrogen receptor-α is present in the testes and epididymides of FST transgenic mice* Estrogen receptor-α (ER-α)-null mice exhibit morphological defects in the efferent ductules similar to those observed in the FST transgenic mice [49-51]. To determine if ER expression was maintained in the presence of elevated FST, we performed IHC for ER in both the testes and epididymides of WT and MMTV-*Fst* mice. ER was abundantly present in the Leydig cells of the testes of FST transgenic mice (Figures 8A). While ER was also distinctly present in the epididymis of MMTV-FST and WT mice, levels were highly variable. In addition, the qualitative nature of IHC precluded determining if the level of expression was altered in various regions of the epididymis between the mouse strains. To determine if the level of *Esr1* mRNA was altered in the entire epididymis of transgenic males, qRT-PCR of total mRNA was used. This revealed that *Esr1* mRNA levels were diminished by 2-fold in the two transgenic lines examined (Figure 8B). In addition, *Esr1* mRNA was downregulated in the efferent ducts of FST overexpressing mice (Supplemental Figure S4A) despite being unchanged or slightly elevated in juvenile mice at days 20 and 24 (Supplemental Figure S4B-C) These data suggest that ER-α is suppressed by FST overexpression in the principal cells of the epididymis and efferent ducts and its dysregulation may, in part, contribute to infertility of FST transgenic males.

## DISCUSSION

While overexpression of FST or FSTL3 in the testes leads to testicular dysfunction and infertility, there are no studies to date examining the functional role of FST or its preferred target, activin, in the epididymis. The MMTV-*Fst* mice described herein have elevated FST in the testis and epididymis and the lesions noted in these mice suggest either: a) a disruption of resorption of fluid entering the efferent ductules from the rete testis, b) an impairment of rhythmic segmental contractions beginning in the initial segment, or c) a distal blockage of sperm transit. The ultrastructural changes in the efferent ducts as well as the diminished expression of *Esr1* indicate a disruption of fluid resorption in the efferent ducts. To our knowledge, this is the first demonstration that FST regulates fluid resorption in the efferent ducts of mice.

Not surprisingly, male mice with overexpression of FST driven by the metallothionin promoter (herein referred to as MT1-*Fst*) yielded a slightly different phenotype than the MMTV-*Fst* mice described here [25]. MT1-*Fst* mice were smaller and had shiny, irregular fur while the MMTV-*Fst* mice were indistinguishable from wild-type littermates. Both MMTV-*Fst* and MT1-*Fst* males had reduced testicular weights in some of the lines although only one of the 5 MT1-*Fst* transgenic lines had 100% infertility of male offspring. In addition, FSH levels were only slightly dampened in one of the MT1-*Fst* lines while FSH levels were in the normal to elevated range in the MMTV-*Fst* mice. These discrepencies can largely be explained by the pattern of expression of the different promoters that were used to create each of these models. Not only did MT1-*Fst* mice have FST overexpression in the Sertoli cells but also had robust expression from the liver and skin. High systemic levels of FST were indicated by the smaller size and fur defects of these mice. In contrast, MMTV-*Fst* mice targeted transgene expression to the Leydig cells, but also in the efferent ducts and epididymides. Although the transgene also resulted in high expression of FST in additional tissues, no changes in weight or fur were observed, indicating that the FST expression did not have broad-ranging effects on the mice. It is possible, but unknown whether the MT1-*Fst* mice also had overexpression in the epididymides. If so, this may have also contributed to the testicular degeneration described in those mice.Similar testes and epididymal phenotypes have been described in mice null for full length *Esr1* as well as truncated and mutated forms of its associated protein [49, 50, 52-54]. These mice are also infertile, have similar testicular lesions and retain fluid in the efferent ducts. Expression of ER-α begins around embryonic day 16 in the epididymis and is expressed throughout adulthood to varying degrees with its highest expression in the efferent ducts [55]. Tracer studies conducted in *Esr1* knockout mice demonstrated that the epithelial cells were capable of endocytosing luminal contents of the efferent ducts, however the total area of endocytotic apparatus was significantly reduced [56]. Although there is no reported evidence for direct regulation of ER-α by FST, activin does induce expression of both the *Esr1* and *Esr2* (ER-β) genes in granulosa cells and this is reversed by treatment with FST [57]. In this context, activin A activation of the *Esr1* promoter is dependent upon Smads 2 and 3. Conversely, the same dose of activin A causes a decrease in *ESR1* expression in MCF-7 breast cancer cells at 24 hours [58] implying that activin regulation of ER transcription may be cell-type dependent. While ER-α is present in the epididymis of the FST mice, its expression is reduced. This reduction may impact expression of solute or fluid transporter target genes such as SLc9a3, or Aquaporins 1, 5 & 9, all of which contribute to fluid homeostasis in the epididymis [59-61] and have been proposed to be direct or indirect targets of ER-α. In addition, FST inhibits AQP5 expression in organ cultures of the submandibular gland and AQP5 is upregulated by estradiol treatment of the efferent ducts [59, 62, 63]. Whether AQP5 is downregulated in the *Esr1* knockout mice has not yet been reported. Of note, AQP1 and 9 are expressed either on the apical or basolateral surface epididymal epithelium, while AQP5 is co-localized to endosomal membranes [64]. Future studies are necessary to determine if FST regulates expression of AQP5 in the epididymis and whether such regulation is modulated by ER.

While we expect that the reduction in ER levels observed in FST mice is due to specific inhibition by FST, it is also possible that ER is subject to autoregulation. Pharmacological levels (75µg/ml) of systemic estradiol in castrated mice can decrease ER-α to undetectable levels in the efferent ducts while exogenous administration of testosterone or dihydrotestosterone has no effect [65]. Although we did not determine the circulating estradiol levels in FST mice, it is unlikely that estradiol is elevated to this level to achieve ER-α down-regulation. If estradiol levels were within this pharmacological range, additional disruptions of the hormonal milieu would be anticipated. Similarly, if FST was significantly elevated in systemic circulation, disruption of the hypothalamic/pituitary/testis axis should occur due to systemic inhibition of activin. However, we found that most of the FST transgenic mice displayed hormonal profiles similar to WT mice. This suggests that the consistent infertility observed in these animals is unlikely to have resulted from a systemic disruption of the hormonal milieu. Further supporting this notion, the MMTV-*Fst* mice displayed no overt abnormalities in tooth development or irregularities in the hair, which has been reported for other models in which systemic overexpression of FST was achieved [25, 66]. While not directly assessed in this study, it is also possible that FST increases intra-testicular levels of estradiol without increasing systemic levels and this, in turn, results in high local concentrations of estradiol in the epididymides.

The propensity for FST to bind and inhibit activin suggests that activin may be critical for normal epididymal function. However, FST can also bind and inhibit myostatin and several BMP family members. Myostatin expression is primarily restricted to skeletal muscle and adipose tissue in adults and homozygous null mice are viable and fertile [67]. Hence myostatin is unlikely to be involved in the infertility defect observed in the MMTV-*Fst* mice. Of the many BMP ligands that FST has been shown to block, only BMP7 is found in the epididymis and its expression is restricted to the initial segment in the mouse after puberty [68, 69]. BMP7 null mice die perinatally and heterozygous male mice are fertile, with no obvious gonadal lesions [70, 71]. Therefore, it is also unlikely that FST blockade of BMP7 alone would cause male infertility. While FST has not been shown to block the activity of BMP8a, it is one of the members of the 60A subgroup of the BMP family, along with BMPs 5, 6, and 7 and many members of this subgroup can be inhibited by FST [72]. BMP8a null mice do have a moderate disruption in the epididymal epithelium. However, all homozygous mutant males were fertile initially, with only 2 of 16 becoming infertile with increasing age [68]. Hence, as with BMP7, FST inhibition of BMP8a alone would not yield the extreme infertility phenotype that is observed in the MMTV-*Fst* mice. Additional disruption of one allele of *Bmp7* within BMP8a null mice results in infertility in approximately 75% of the mice [69]. The compound mutants display a similar phenotype to MMTV-*Fst* mice with degeneration of the epididymal epithelium along with an accumulation of sperm and subsequent obstruction of the lumen of the initial segment of the epididymis. Interestingly, the infertility phenotype in compound mutants is dependent upon the C57BL/6 genetic background and does not occur in mice within a mixed genetic milieu. The MMTV-*Fst* founder mice described herein are in a combined genetic background of C57BL/6 and SJL mouse strains, while the F1 generation has genetic contributions from the C57BL/6, SJL and FVB/N strains. The penetrance of the infertility phenotype remains constant in MMTV-*Fst* males, despite the differences in genetics between the founder and F1 generation. This underscores the importance of FST regulation of fluid resorption in the efferent ducts independently of genetic predisposition and supports the notion that inhibition of all signaling from activin in addition to BMP7 and BMP8a may underlie the profound phenotype in MMTV-*Fst* males.

While FST has not been demonstrated to bind to mammalian BMP8b and BMP4, both factors also impact the epididymis. BMP8b, is only expressed in the testis, yet inactivation of the *Bmp8b* gene also has a functional consequence in the initial segment of the epididymis suggesting that BMPs as well as activins may serve as luminal fluid cytokines to maintain function of the rete testes and excurrent ducts [68]. BMP4 is expressed ubiquitously in the epididymis throughout postnatal development and while homozygous null mice die in utero, heterozygous male mice are subfertile/infertile when bred into the C57BL/6 background [73]. These mice have reduced sperm counts and motility along with compromised epithelial integrity of the corpus epididymis. It is unclear if FST can bind and inhibit the function of BMP4 in mammals [10, 74, 75] however, in *Xenopus* the antagonistic affects of FST on BMP4 have been well established [9, 75]. Although disrupting the expression of individual BMPs or activin from the epididymis leads to mild to moderate infertility in mice, the fact that FST overexpression in a mixed genetic background leads to a catastrophic loss of fertility and epididymal epithelial integrity suggests that FST may also inhibit additional BMP function in the efferent ducts.

In MMTV-*Fst* seminiferous tubules that retain normal morphology, spermatogenesis appears to proceed normally, even in the presence of overexpressed FST in the testes. Similar results were seen in the testes of transgenic mice with FST driven by the metallothionin promoter [25]. This suggests that FST overexpression does not directly cause sperm abnormalities in these mice. While the data presented herein support the postulate that increased pressure/tension in the epididymal lumen leads to mechanical shear forces during sperm transport, there are other factors that could contribute to the observed sperm defects. Sperm chromatin abnormalities can be induced by increasing the temperature of the scrotum to 38°C and sperm is altogether eliminated in the cauda epididymis if internal temperatures reach 42°C [76]. In addition, luminal acidification is necessary for sperm maturation and maintains immature sperm in an immotile, quiescent state. Upon admixture with the more alkaline prostatic fluid, motility is restored to achieve maximum mobility at ejaculation [77-79]. Elevation of the luminal pH of the epididymis results in male infertility due to the inability of sperm to reach the female genital tract [80]. In the *Esr1* knockout mice, sperm defects were attributed to an increase in pH in addition to altered osmolality of the lumen which resulted in reduced motility and severe flagellar coiling (also known as the Dag defect) [81, 82]. However, the primary sperm defect in MMTV-*Fst* mice is not the result of immotility due to premature exit from quiescence, but separation of the head from the tail. Mice with FST, FSTL3 or Activin C overexpressed in the Sertoli cells have reduced sperm numbers and sperm motility. In contrast, we found that MMTV-*Fst* mice overexpress FST in the Leydig cells. This permits normal spermatogenesis in the testes and sperm defects are secondary to the efferent duct blockade [24-26]. Further studies are necessary to elucidate whether the sperm defects in the MMTV-*Fst* mice are restricted to detachment of heads consequent to fluid build-up resulting in degeneration, or if the sperm also suffer from additional deficits not apparent in the assays performed herein.

The ability of FST to induce sperm decapitation and infertility raises the possibility that FST could serve as a male contraceptive. The guiding axiom of current hormonal contraceptive research is to abrogate LH and FSH to inhibit spermatogenesis while at the same time supplementing with testosterone to maintain libido [83]. MMTV-*Fst* males manifest infertility while testosterone levels and copulatory behavior are maintained, suggesting no impact on male libido, making FST an ideal candidate for male contraception. Further studies are necessary to determine the threshold and timing of epididymal FST that is necessary to induce infertility as well as the reversibility of its effects.

In summary, we show for the first time that male reproductive fitness is dependent upon a balance of activin and/or BMP signaling that is modulated by FST in the efferent ductules of the epididymis. Dysregulation of this equilibrium leads to fluid retention and ensuing pathologies of the seminiferous epithelia that culminate in abnormalities of sperm transport and chromatin condensation. This ultimately causes sperm decapitation and complete infertility. FST inhibition of either activin, BMP or both may be critical for proper epithelial integrity, contractility and vesicular trafficking of the epididymis. Whether or not FST alters *Esr1* expression through activin or BMP inhibition in the epididymis is still unclear. Further analysis of the contributions of ER-α downregulation to the infertility phenotype of MMTV-*Fst* mice is necessary to determine if this is the primary mechanism by which FST causes infertility.

**Acknowledgements**
The excellent technical expertise of Kristen Lozada in mouse husbandry and Carol Moeller and Jennifer Palmer in transmission electron microscopy are gratefully acknowledged. We also recognize Gina Bernardo for assistance in editing the manuscript. The Case Transgenic and Targeting Facility generated MMTV-*Fst* transgenic mice and the Tissue Procurement, Histology and Immunohistochemistry core facility of the Case Comprehensive Cancer Center (P30 CA043703) and ARBL Morphological Services at Colorado State University provided technical services associated with histopathology and microscopy.

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## Figure Legends

**Table 1**. *Detailed summary of mating experiments and outcomes.* Number of mice used per breeding are displayed parenthetically.

**Figure 1.** Schematic of the breeding paradigms used to assess infertility in MMTV-*Fst* males. **A**) Breeding paradigm used to determine infertility in founder males. Curved arrows indicate

that proven females replaced females that had not yielded litters. **B**) Breeding paradigm used to determine that the male infertility phenotype was heritable and limited to males.

**Figure 2.** Many infertile MMTV-FST males have serum hormone levels and testes and seminal vesicle weights that are identical to WT males. **A**) Serum testosterone levels in WT (n=16) and MMTV-*Fst* (n=17) male founder mice (*p<0.05). **B**) Serum FSH in WT and MMTV-*Fst* F1 offspring from transgenic lines B and C. WT littermates from both transgenic lines are combined (n=14), FST B (n=6; **p<0.01 ), FST C (n=4). **C**) Serum LH in WT and FST F1 offspring from transgenic lines B and C. WT littermates from lines B and C are combined (n=14), FST B (n=6; *p<0.05), FST C (n=4). **D**) Testes weight relative to body weight in FST lines A (n=3), B (n=5; ***p<0.001) and C (n=6; *p<0.05) and WT littermates combined from all 3 lines (n=20). **E**) Seminal vesicle weight relative to body weight in FST lines A (n=3), B (n=5; *p<0.05) and C (n=6) and WT littermates combined from all 3 lines (n=20).

**Figure 3.** FST overexpression occurs in the Leydig cells of the testes and principal cells of the epididymis of MMTV-*Fst* mice. **A**) Quantitative Real-Time PCR from RNA isolated from whole testes comparing WT (n=13) and FST offspring from Lines A (n=4), B (n=4) and C (n=4). ***p<0.001 for all 3 lines compared to WT controls. **B**) Quantitative Real-Time PCR of RNA isolated from epididymides comparing littermate WT (n=6) and FST Lines A (n=3; ***p<0.01) and B (n=3; *p<0.05). **C**) Immunohistochemistry utilizing anti-FST antibody reveals FST overexpression (brown stain) is localized to Leydig cells of the testes and principal cells of the epididymis. Original magnification for all images x200.

**Figure 4.** FST overexpression leads to a spectrum of testicular lesions consistent with a fluid reabsorption defect in the excurrent ducts. **A-B**) Representative 1-µm-thick testicular sections from WT (**A**) and MMTV-*Fst* (**B**) mice displaying normal morphology. **C-F**) A variety of lesions are evident in the epithelium of seminiferous tubules and rete testis of transgenic MMTV-*Fst* mice. Extensive vacuolation in seminiferous epithelium (**C;** arrows), interstitial edema (**D**), fluid-engorged rete testis with denuded seminiferous epithelial elements in two rete tubules and sperm stasis in adjacent seminiferous tubule cross sections (**E**), and seminiferous epithelial vacuolation, sperm stasis and mineralization (**F**). Toluidine blue staining. Scale bars = 40 µm (**A**, **B**, **D,** and **F**) and 20 µm (**C** and **E**).

**Figure 5.** Fluid resorption and sperm transit are impaired in the testicular excurrent ducts of MMTV-*Fst* mice. Representative 1 µm-thick sections of the efferent ducts (**A-B**), caudae epididymidis (**C-D**) and distal corpora/proximal caudae epididymidis (**E-F**). A,C: WT. B,D,E,F: MMTV-*Fst*. In contrast to normal morphological features of efferent ducts (**A**) and cauda epididymidis (**C**) in the WT mice, accumulation of sperm in the efferent ducts is evident in MMTV-*Fst* mice (**B**); degenerate prematurely shed germ cells are seen both in the efferent duct (**B**) and also in the lumina of cauda epididymidis (**D**) and the distal corpus/proximal cauda **(E, F**). Tail-intact sperm are sparse in these epididymal regions (**D-F**) of MMTV-*Fst* mice. An adenomyotic outpouching of epithelial lining, or cystic formation (arrow in **B**) is seen in this section of efferent duct from a MMTV-*Fst* mouse. Toluidine blue staining. Scale bar = 20 µm.

**Figure 6.** FST overexpression results in decreased sperm motility consequent to detachment of sperm heads and tails and also causes ultrastructural defects in sperm nuclei. **A**) Differential

interference contrast images of sperm harvested from caudae epididymidis from WT (left) and MMTV-*Fst* (right) mice. Bars = 20µm. **B**) Graph showing incidence of detached sperm heads (***$p<0.001$). **C**) Transmission electron micrograph of sperm showing defects in chromatin condensation (arrow) and nuclear craters (arrowheads). Scale bars = 20 µm (**A**) and 2 µm (**C**).

**Figure 7.** Transmission electron micrographs of efferent ducts in WT and MMTV-FST mice. WT (**A-C**), MMTV-FST (**D-F**). **A, D**) In contrast to the normal non-ciliated (NC) and ciliated (CL) epithelial cells in the WT efferent duct, the ultrastructural organelles of these cells in the FST mouse appear to be compacted and abnormal even at this low magnification. The boxed areas of representing apical and basal aspects of the epithelium are magnified in **B, C** and **E, F**, respectively. Whereas the lumen is clear and apical specializations—microvilli and cilia—appear freely protruding into the lumen in the WT (**A**), these structures are compressed by the accumulated luminal contents in the FST efferent duct (**D**); a longitudinal section of a sperm head is seen. **B, E**) An elaborate endocytotic apparatus consisting of coated pits and tubulovesicular system (asterisk) is seen in the WT (**B**) whereas this apparatus is loaded with cellular debris and phagosomes are clearly seen (arrows) in the FST duct (**E**). The apical tight junctions between the epithelial cells (arrow) do not appear to be compromised in the FST mouse. **C, F**) On the basal aspect, the basal laminae are abnormally thickened in FST (**F**) compared to WT (**C**). In the lamina lucida, the zone closest to the epithelium (double-headed arrow), FST mice exhibit abnormal inclusions (asterisks). The lamina densa underlying the lamina lucida (bracket) is also thickened in FST whereas it is barely discernible in the WT duct. These abnormally thickened basal laminae impede transcytosis of luminal fluid toward the lymphatics and microcirculation in the lamina propria. Scale bars = 1 µm (**A**,**D**) and 500 nm (**B**,**C**,**E**,**F)**.

**Figure 8**. ER-α expression is downregulated in the epididymis as a consequence of FST overexpression. **A**) Representative images of immunohistochemistry utilizing antibody against ER on sections from the testes and epididymides of WT and MMTV-*Fst* mice. Original magnification of images x200. **B**) Quantitative Real-Time PCR from RNA isolated from total epididymides of WT (n=7) and FST Line A (n=3, *$p<0.05$ compared to WT) and Line B (n=4, *$p<0.05$ compared to WT).

Seachrist, Darcie et al. Table 1

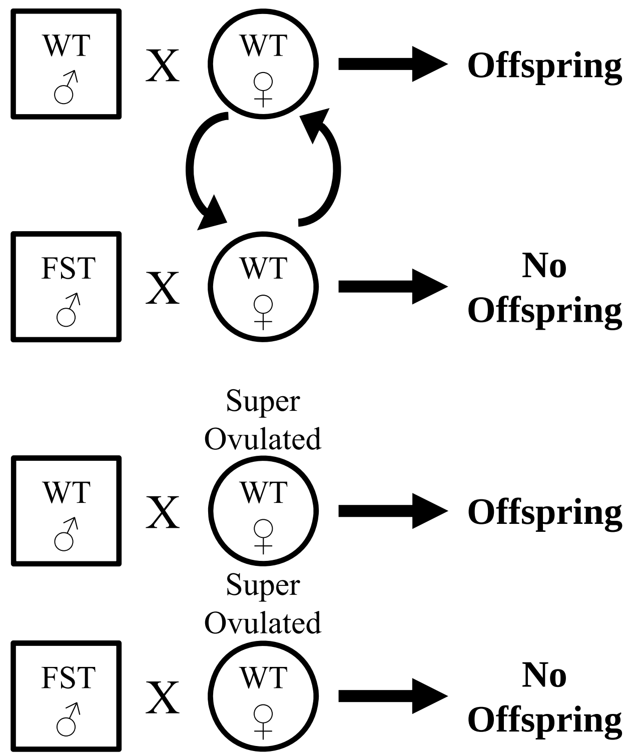
Table 1.
*Detailed summary of mating experiments and outcomes.*

| Mating description | No. males producing offspring | Percent fertile males |
|---|---|---|
| WT♂xVir♀ (n=5) | 5 | 100% |
| FST♂xVir♀) (n=17) | 1 | 6% |
| WT♂xSO♀ (n=5) | 5 | 100% |
| FST♂xSO♀ (n=5) | 0 | 0% |
| WT♂xProven♀ (n=5) | 5 | 100% |
| FST♂xProven♀ (n=5) | 0 | 0% |

(Vir=virgin; SO=super-ovulated; Proven=proven breeder) Number of mice used per breeding are displayed parenthetically.

Seachrist, Darcie et al. Figure 1

A

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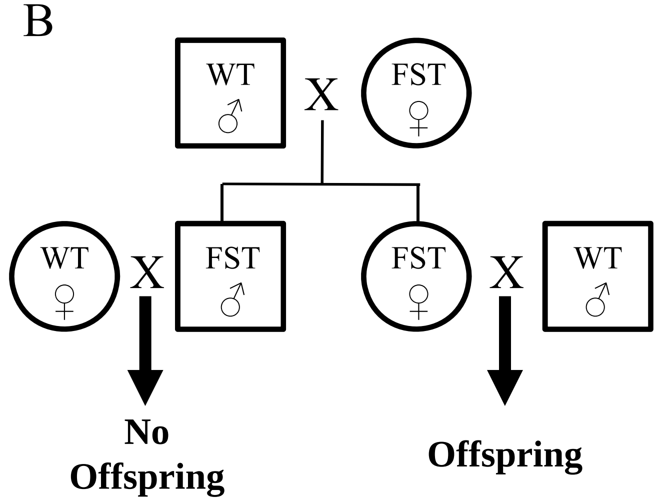
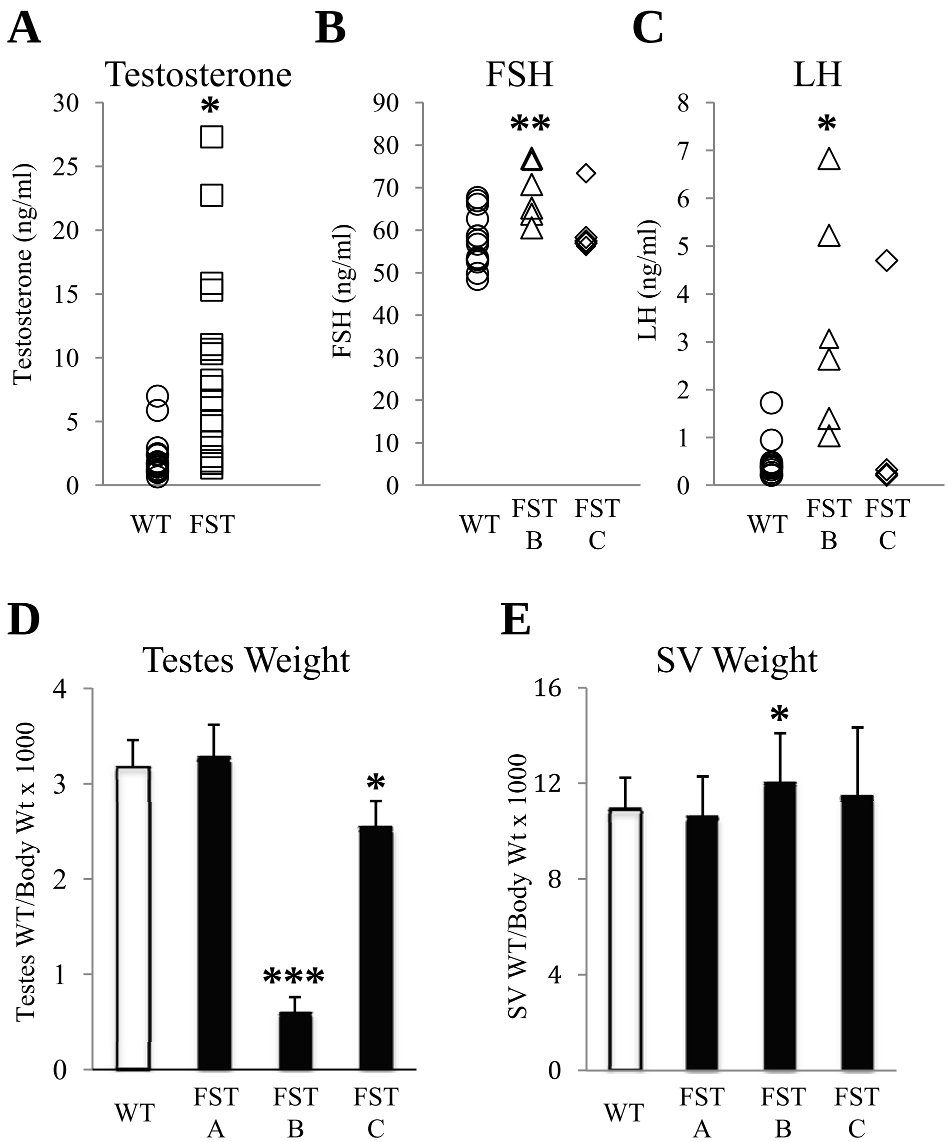
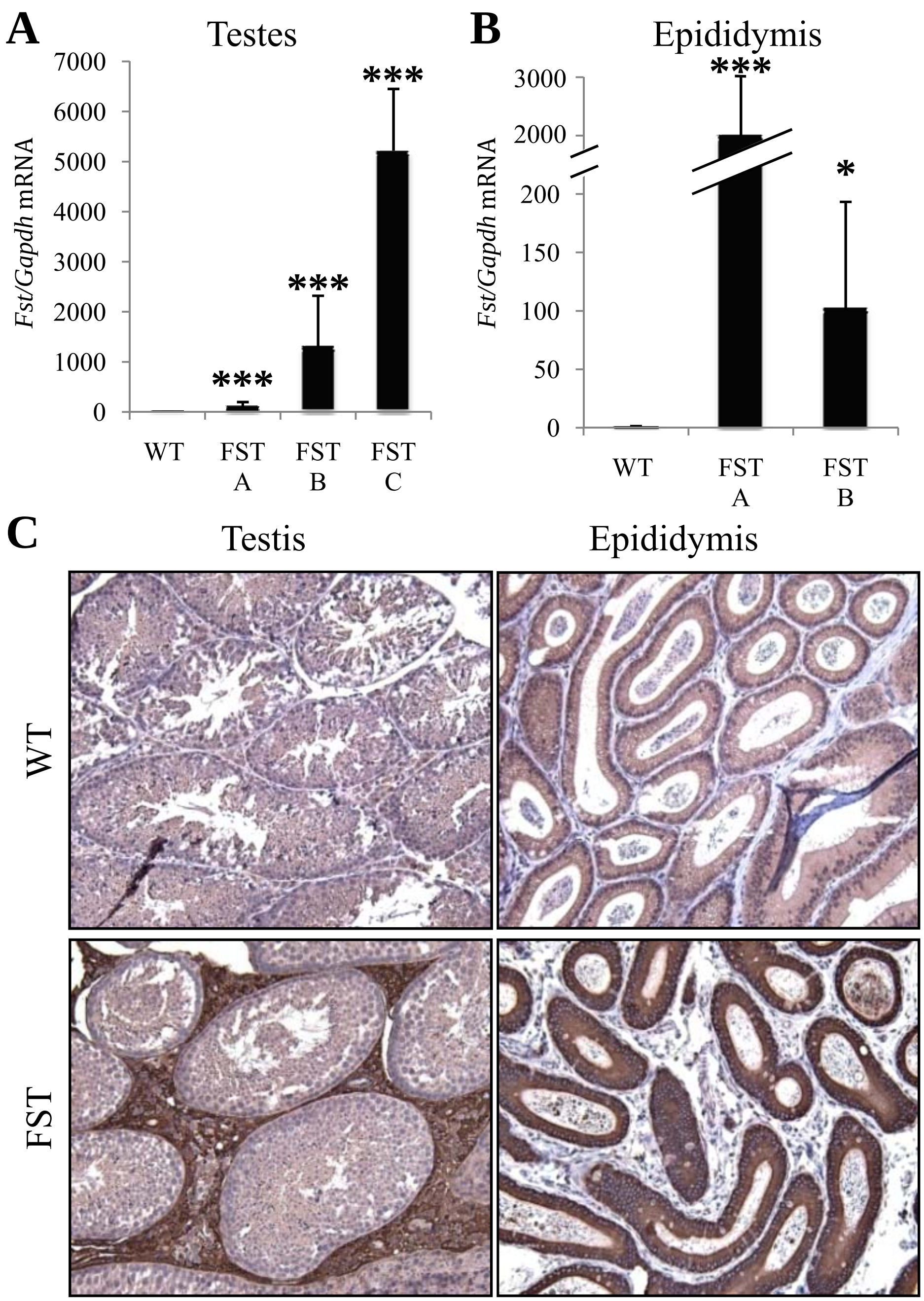


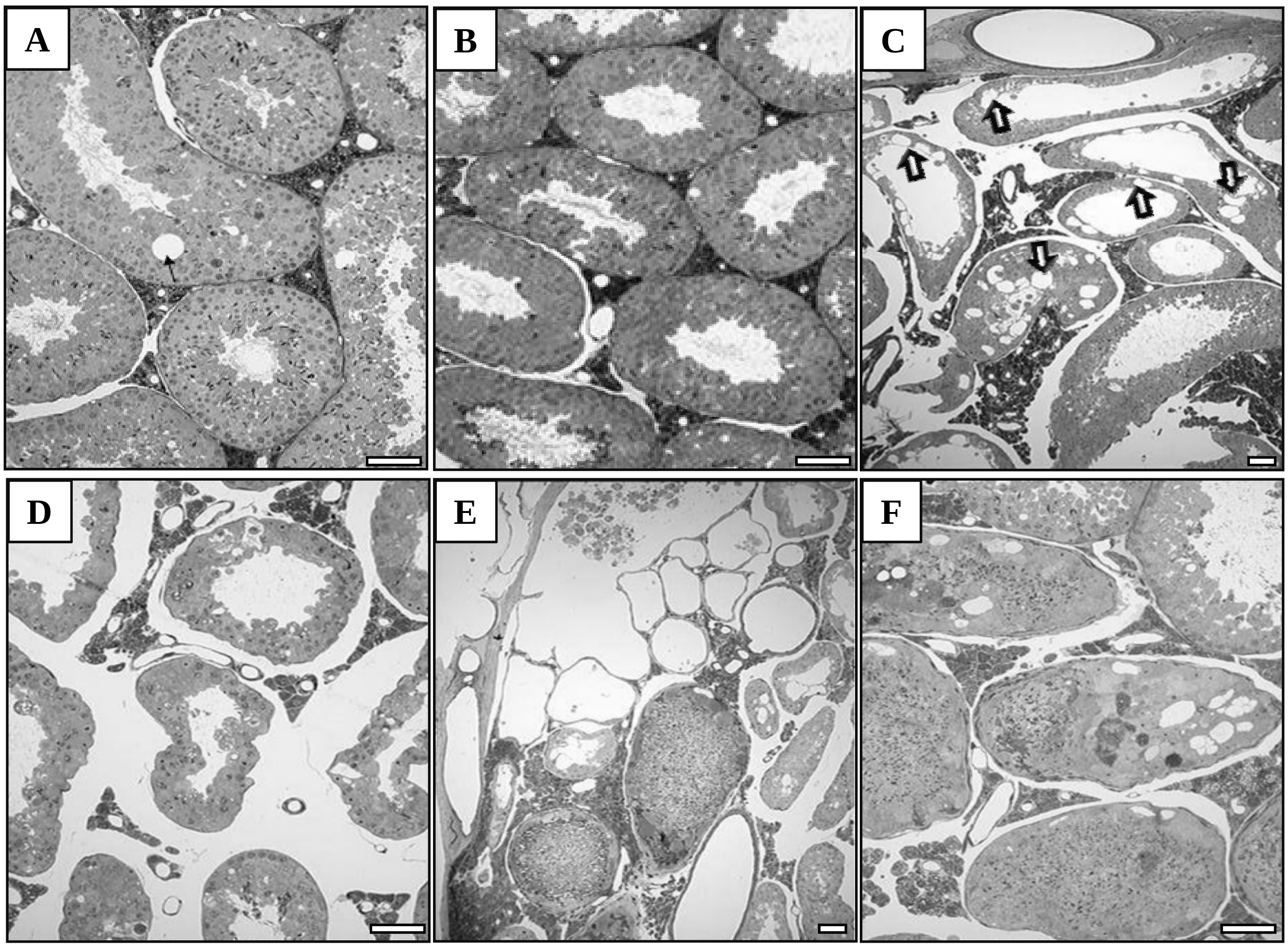
Seachrist, Darcie et al. Figure 2



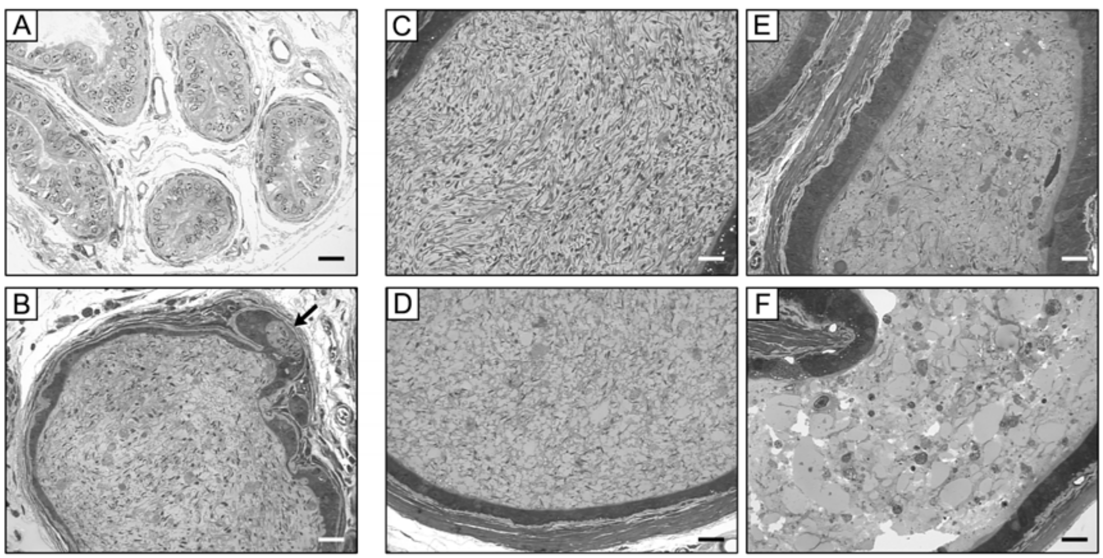
Seachrist, Darcie et al. Figure 3



Seachrist et al. Figure 4



Seachrist et al. Figure 5



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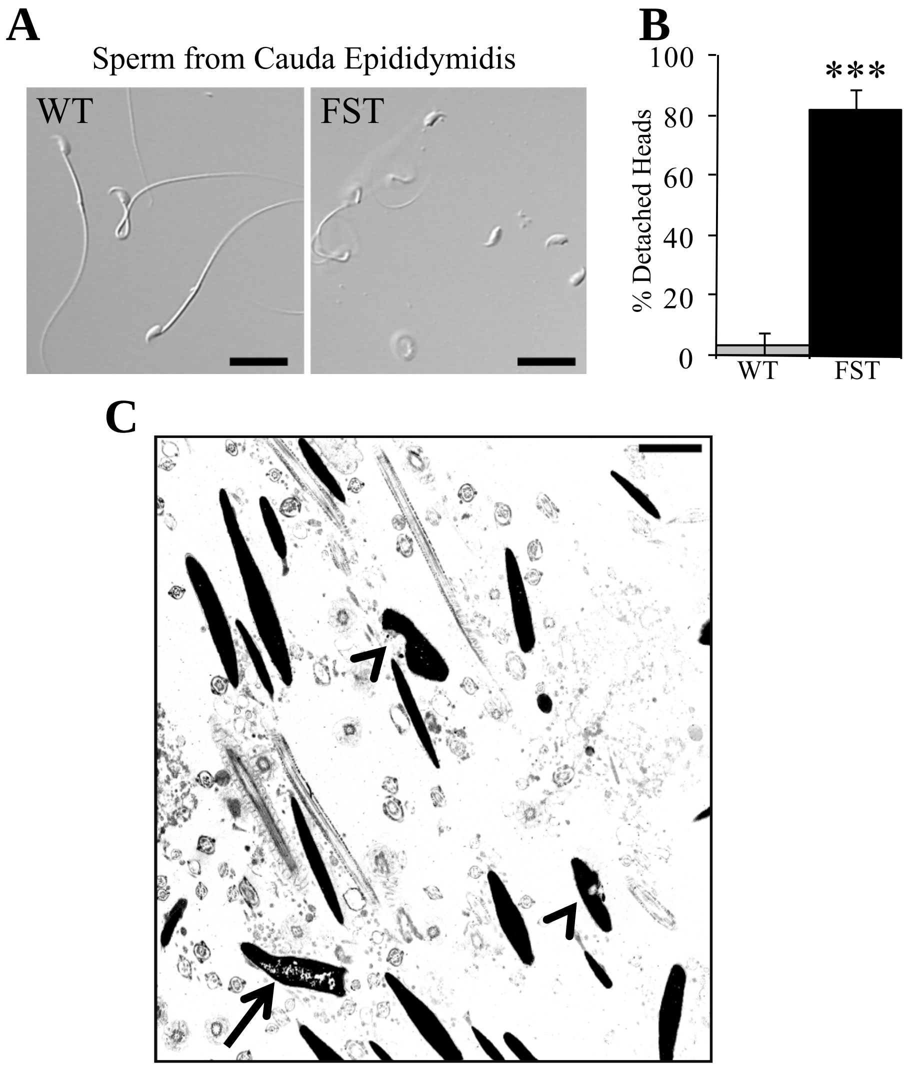
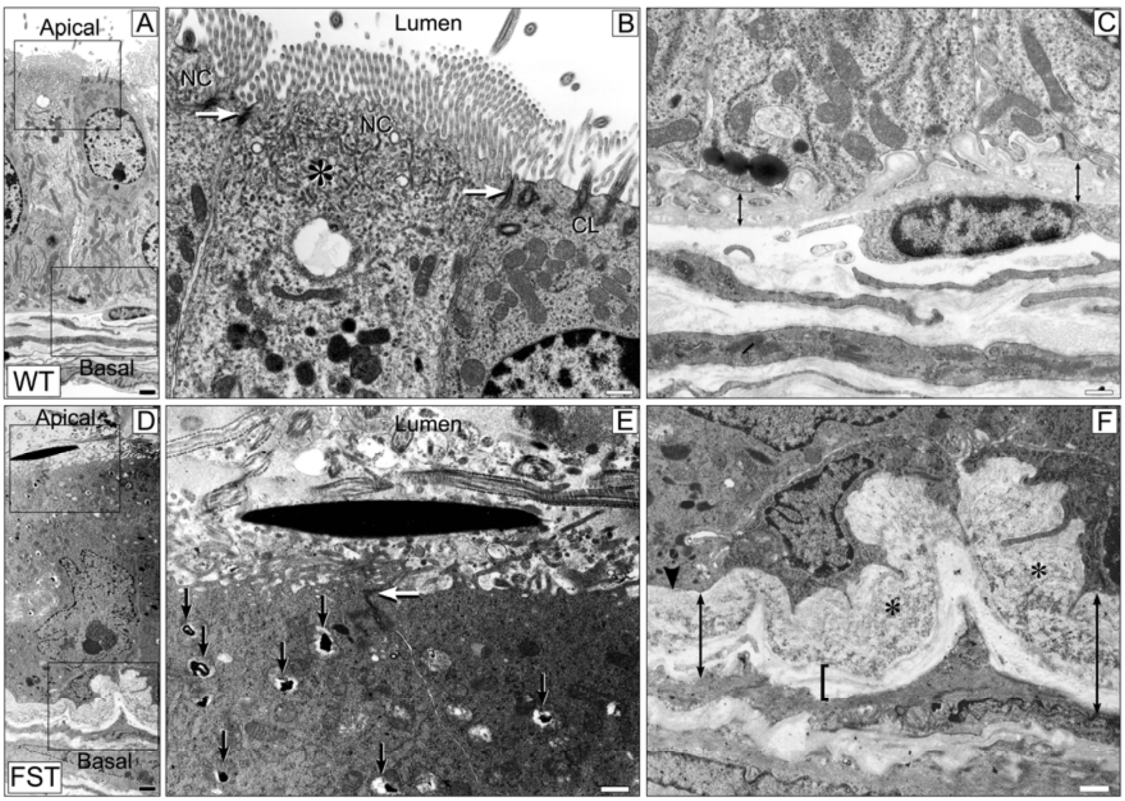


Seachrist et al. Figure 7



Seachrist, Darcie et al. Figure 8