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Male Reproductive Phenotypes in Double Mutant Mice Lacking Both FSHβ and Activin Receptor IIA

T. RAJENDRA KUMAR, SIMONA VARANI, NIGEL G. WREFORD, NANCY M. TELFER, DAVID M. DE KRETSER, AND MARTIN M. MATZUK

Departments of Pathology (T.R.K., S.V., M.M.M.), Molecular and Cellular Biology (T.R.K., M.M.M.), and Molecular and Human Genetics (M.M.M.), Baylor College of Medicine, Houston, Texas 77030; and Department of Anatomy (N.G.W., N.M.T.) and Monash Institute of Reproduction (D.M.D.) Monash University, Melbourne, Victoria 3168, Australia

Activins are known to signal through two serine/threonine kinase type II receptors. Activin receptor IIA is widely expressed in the male reproductive axis, including the pituitary and testis. Our previous studies using gene knockout mice have confirmed the essential *in vivo* role of activin receptor IIA in FSH homeostasis. Activin receptor IIA-null male mice are fertile, have suppressed pituitary and serum FSH levels, and demonstrate a decrease in testis size as a result of reduced Sertoli cells and germ cells. Similarly, FSHβ null male mice are fertile despite reduced testis size and Sertoli cell number. To define the direct roles of activin receptor IIA signaling locally in the testis, independent of its effects on FSH homeostasis, we generated double mutant mice lacking both activin receptor IIA and FSH by a genetic intercross and analyzed the male reproductive phenotypes. The double mutant male mice lacking both FSH and activin receptor IIA are fertile, demonstrate no significant reduction in testis size, and produce small litters compared with mice lacking either FSH or activin receptor IIA alone. Histological analyses of the testes from double mutant mice revealed the presence of normal stages of spermatogenesis. However, there was a significant reduction in the epididymal sperm number compared with that of the individual mutants. Northern blot analyses of total RNA from testes of double mutants did not reveal transcriptional up-regulation of activin receptor IIB, the other activin type II receptor. Although RNA expression profiles of many testis cell-specific markers are unaltered, stereological analysis of the testes from double mutants indicates that there was a reduction in type A and I spermatogonial number compared with that observed in individual mutants. Our results provide *in vivo* genetic evidence to demonstrate that activin receptor IIA signaling plays an important local role within the testis, independent of its actions via FSH homeostasis in the pituitary. (*Endocrinology* 142: 3512–3518, 2001)

FSH IS A pituitary-derived heterodimeric glycoprotein hormone. The α-subunit is shared among other members of the family, LH and TSH, whereas the β-subunit is hormone specific (1, 2). The noncovalent interaction between the two subunits is required for biological activity of each of these hormones (1, 2). Expression of the FSH β-subunit is positively regulated by the activins (3, 4). FSH receptors are localized to Sertoli cells within the testis, and FSH is a well known mitogen for Sertoli cells (5, 6).

Activins are members of the TGFβ superfamily, synthesized as precursor proteins and cleaved to mature forms before secretion (3, 4). They were originally discovered as homo- or heterodimeric gonadal peptides involved in positively regulating FSH biosynthesis and secretion from the pituitary (7, 8). Physiologically, three types of activins exist: activin A (β_A:β_A), activin B (β_B:β_B), and activin AB (β_A:β_B); however, activins A and B are the most characterized activins (3, 4). Activins are also expressed in multiple tissues outside the reproductive axis, where they act in both an autocrine and paracrine manner (9, 10).

Within the reproductive axis, activin B is predominantly expressed in the pituitary, whereas both activins A and B are expressed in Sertoli cells of the testis (11). Several *in vivo* and *in vitro* studies have shown that activins influence testis development and function directly or indirectly (12–16). However, it is not known whether activins can act directly within the testis *in vivo* independently of their effects on FSH homeostasis.

Activins bind to two types of serine/threonine kinase receptor isoforms, type IIA and type IIB (ActRIIA, ActRIIB), and the genes encoding them are localized to distinct chromosomes (17, 18). ActRIIA is more ubiquitously expressed than ActRIIB, is evolutionarily conserved, and is the major component in the activin signaling pathway in the adult (17–19). Rat, mouse, and human ActRIIA are also localized to pituitary gonadotropes and Sertoli cells in the testis, similar to activins A and/or B (20). Additionally, ActRIIA is localized to germ cells, in particular to pachytene spermatocytes and A-type spermatogonia, in the testis of rats and mice (21, 22). The coordinated expression of the activin ligands and their type II receptors to multiple cell types within the testis suggests that they may act locally in an autocrine/paracrine manner.

Gene knockout studies from our laboratory have previously confirmed the *in vivo* role of ActRIIA signaling in the mouse reproductive axis (23). The majority of ActRIIA-null mice are viable (~22% die embryonically), and demonstrate reproductive defects (24). Mutant males are fertile, have suppressed serum and pituitary FSH levels, and demonstrate a decrease in testis size (23). It is not known whether these phenotypes are secondary to suppressed FSH levels or are a direct result of impaired ActRIIA signaling locally within the

Abbreviations: ActRIIA, Activin receptor IIA; FAR, FSHβ/ActRIIA double homozygotes; WT, wild-type.

testis. Previously, we have also generated FSHβ-null (and hence FSH-deficient) mice to study the consequences of isolated FSH deficiency in reproductive physiology (24). Similar to ActRIIA-null male mice, FSHβ-null male mice are fertile and demonstrate reduced testis size and reduced epididymal sperm number and motility (24).

To examine the local role of ActRIIA signaling within the testis independent of its effects on FSH homeostasis, we initially intercrossed FSHβ-null males and ActRIIA heterozygous female mice (ActRIIA-null females are infertile) and eventually generated FSHβ/ActRIIA (FAR) double homozygous mutant male mice. Here, we report the male reproductive phenotypes in these double mutant mice that lack both FSH and ActRIIA.

Materials and Methods

Generation of FAR double mutant mice and fertility analysis

Matings between FSHβ-null males and ActRIIA-heterozygous females were initially performed to obtain double heterozygous mice. The double heterozygous mice were intercrossed to generate FAR double mutant mice. The ActRIIA and FSHβ mutant alleles were diagnosed by appropriate restriction enzyme digestions of the tail DNA followed by probing the Southern blots with radiolabeled external probes as described previously (23, 24). Adult FAR double mutant mice at 42 d of age were caged with double heterozygous female mice (one female per male, total of six pairs) for fertility analysis over a period of 1 yr. The numbers of litters and litter sizes were recorded and used to calculate the breeding performance. All mice were of the C57BL/6/129SvEv hybrid genetic background and maintained according to NIH guidelines adopted by Baylor College of Medicine.

Morphological and histological analysis

Testes were collected from adult mice at 42 d [five wild-type (WT) and five FAR double mutant mice] and weighed. For histological analysis, testes samples were fixed in Bouin's reagent overnight at room temperature and thereafter rinsed extensively for 48 h in $LiCO_3$-saturated 70% ethanol (23, 24). Paraffin-embedded 4-μm sections were later stained with periodic acid-Schiff/hematoxylin as previously described (23, 24). Apoptotic cells were stained using a variation of the terminal transferase-mediated deoxy-UTP nick end labeling method as described by Gavrieli *et al.* (25). The labeled DNA was detected using an alkaline phosphatase-labeled sheep antidigoxigenin Fab conjugated with alkaline phosphatase. The chromogen substrate mixture used was Fast Red TR/napthol (Sigma, St. Louis, MO).

Stereological analysis

Stereological analysis of the testis (from five mice per group) was performed on 20-μm thick methacrylate sections stained with periodic acid-Schiff reagent as described previously (26).

Epididymal sperm quantitation

Epididymides from both sides were collected from mice at 42 d (five or six per group) into 1 ml M2 (modified Whitten's) medium (PGC Scientifics, Gaithersburg, MD) to release sperm after a 15-min incubation at 37 C. The released sperm were counted using a hemocytometer as previously described (24, 27, 28).

Estimation of intratesticular testosterone content

Freshly isolated testis samples from 42-d-old mice (five or six per group) were decapsulated, and homogenates (1 ml) were prepared according to the method of Meistrich *et al.* (29). The testosterone content in the supernatants was quantitated by a solid phase testosterone RIA (Diagnostics Systems Laboratories, Inc., Webster, TX) according to the protocols provided by the manufacturer using calibrated hormone standards. The values are expressed as nanograms per mg testis. The sensitivity of the assay is 0.08 ng/ml, with an intraassay coefficient of variation of 8.6% and an interassay coefficient of variation of 9.6%.

RNA isolation and Northern blot analysis

Total RNA from testes was extracted (three mice per group) using RNA STAT-60 (Leedo Medical Laboratories, Houston, TX) as previously described (30). Fifteen micrograms of denatured RNA samples were separated on 1.4% agarose gels containing 13% formaldehyde, transferred to nylon membranes, vacuum-baked at 80 C, hybridized with [^{32}P]deoxy-CTP-labeled random primed probes, washed, and exposed to autoradiography films as previously described (31). The autoradiography films were scanned by densitometry, and the ratio of the individual probe signal to 18S signal was calculated and analyzed by one-way ANOVA for statistical significance. Northern blot analysis was performed twice under exactly the same conditions. The cDNA probes were PCR-generated and sequenced to confirm the identity of the sequences, or they were obtained as gifts.

Statistical analysis

All the data are presented as the mean ± SEM and were analyzed by *t* test or one-way ANOVA using an Excel version 6.0 software package (Microsoft Corp., Seattle, WA). $P < 0.05$ was considered statistically significant. Stereological data were analyzed using SigmaStat version 2.0 software (San Rafael, CA). The SE of the ratios were calculated using the formula given by Kendall and Stuart (32).

Results

FAR double mutant male mice have decreased fertility

To generate FAR double mutant mice, we employed a 2-step genetic intercross scheme (Fig. 1) involving initial matings between FSHβ-null male and AtRIIA heterozygous female mice (step 1, Fig. 1). This intercross resulted in double heterozygous male and female mice, which were subsequently intercrossed to generate FAR double homozygous mutant mice (step 2A, Fig. 1). As a result of this genetic cross, we also obtained male mice that were homozygous mutant at the FSHβ locus and heterozygous at the ActRIIA locus, or *vice versa*. These male mice were also mated to double heterozygous female mice (step 2B, Fig. 1) to increase the frequency of generating FAR double mutant mice. Male mice lacking FSHβ or ActRIIA alone are fertile and produced comparable number of pups when mated to the corresponding heterozygous female mice (data not shown) (23, 24). To evaluate the fertility of FAR double mutant male mice, 6 double mutant males were mated to double heterozygous female mice (1 female/1 male) beginning at 42 d over a period of 1 yr. All of the 6 double mutant males were fertile and sired offspring. However, the mean litter size was significantly decreased compared with that from matings between age-matched double heterozygous male and female mice [2.8 ± 0.2 pups (31 litters) *vs.* 5.0 ± 0.1 pups (49 litters); $P < 0.001$]. Although 22% of mice die embryonically due to deficiency of ActRIIA alone, the average litter size from ActRIIA-null male and heterozygous ActRIIA female matings was significantly higher than that obtained from matings between FAR double mutant male and FAR double heterozygous female mice [5.2 ± 0.6 pups (14 litters) *vs.* 2.8 ± 0.2 pups (31 litters); $P < 0.001$]. Furthermore, there were no statistically significant differences between single and double mutants when their average ages at siring the first litters (mated to corresponding heterozygous females) were com-

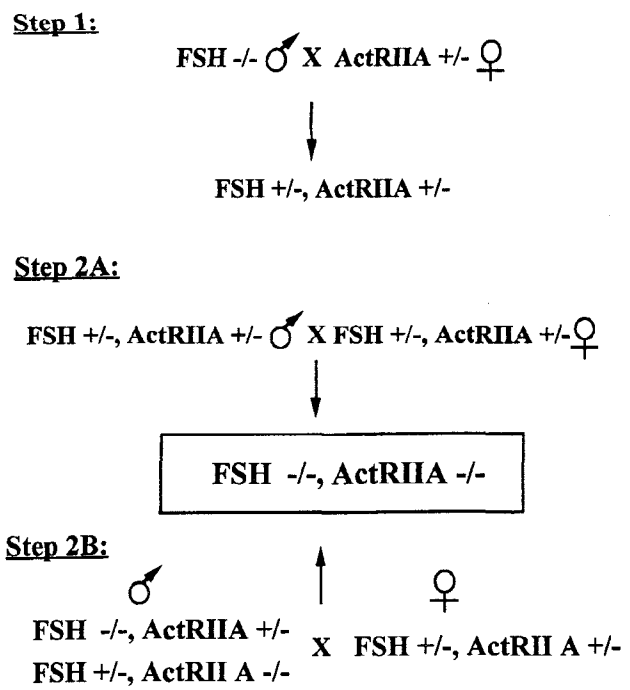


FIG. 1. Strategy for producing FAR double mutant mice. Double mutant mice lacking both FSH and ActRIIA are generated in a two-step genetic intercross scheme. First, FSHβ-null male mice were mated to ActRIIA heterozygous female mice to generate double heterozygous mice. These mice were then intercrossed to generate FAR double mutant mice. Additionally, male mice that were FSHβ-null and ActRIIA-heterozygous or ActRIIA-homozygous mutant and FSH-heterozygous were mated to double heterozygous female mice to generate FAR double mutant mice.

pared ($FSH^{-/-}$, 70 d; $ActRIIA^{-/-}$, 78 d; $FAR^{-/-}$, 73 d; $P > 0.05$; n = 6–7/group). Thus, double mutant male mice lacking both FSH and ActRIIA are fertile, but they produce small litters when mated to double heterozygous female mice.

Reduced epididymal sperm numbers in FAR double mutant male mice

Testis size in individual mutants that lack either FSH or ActRIIA alone is decreased (Table 1) (23, 24) with no apparent effect on the ability to achieve a pregnancy, although litter size is decreased in the double homozygous mutants *vs.* double heterozygous matings at 42 d or at later time points (data not shown). Thus, it is unlikely that the male reproductive phenotypes of double homozygous mutants at 42 d represent a delay in puberty. To further characterize the apparently decreased fertility in FAR double mutant male mice, testicular phenotypes were morphologically, stereologically, and functionally analyzed. First, we measured testis size in FAR double mutant mice at 42 d and compared these to testis size in FSHβ- or ActRIIA-null mice at the same age. As shown in Table 1, the testis size in double mutants was not significantly different from that in FSHβ- or ActRIIA-null mice, although the FAR double homozygous mutant mice lacked both FSH and ActRIIA. Similarly, routine histological analysis of testes from FAR double mutant male mice at 42 d revealed no differences compared with sections from either FSHβ- or ActRIIA-null mice (Fig. 2) (23, 24). At 42 d all of the stages of spermatogenesis appeared normal in the testis of FAR double mutant mice, and there were normal Leydig cell islands present between the tubules (Fig. 2). Although spermatogenesis appeared grossly normal, and there were no differences in epididymal weights (data not shown), epididymal sperm number was significantly reduced in FAR double mutant mice at 42 d compared with the number in single mutants (Table 1) (23, 24). Furthermore, despite the wide variation, there was a trend toward an increase in the mean intratesticular testosterone levels in FAR double mutant mice (Table 2). Together, these results suggest that the decreased fertility in FAR double mutant male mice is due to decreased sperm production.

TABLE 1. Testis weights and epididymal sperm numbers in mutant mice

Genotype		Testis wt (mg)	Epididymal sperm ($\times 10^6$)	No. of mice
FSHβ	ActRIIA			
+/+	+/+	83.2 ± 2.9 (8)[a]	5.2 ± 0.2[e]	6
−/−	+/+	28.2 ± 0.9 (9)[b]	1.3 ± 0.4[f]	6
+/+	−/−	24.1 ± 1.5 (6)[c]	1.5 ± 0.2[g]	6
−/−	−/−	23.7 ± 1.3 (6)[d]	0.4 ± 0.04[h]	6

Adult mice at 42 d were analyzed. Spermatozooa were collected from both epididymides (24, 27, 28). Values are the mean ± SEM in each case. By single factor ANOVA, *a vs. b* or *c* or *d*, $P < 0.001$; *b vs. c* or *d*; *c vs. d*, $P > 0.05$ *e vs. f* or *g* or *h*, $P < 0.001$; *f* or *g vs. h*, $P < 0.001$. +/+, Wild-type; −/−, homozygous mutant.

Stereological analysis of FAR double mutant mice

To determine cell numbers in the testis of FAR double mutant mice, stereological analysis was performed (26, 33). Stereological analysis of the testis from double mutants shows a similar profile to the individual knockouts (34). Sertoli cell number was reduced by approximately 30% compared with that in WT mice (Fig. 3), which is very similar to the 30% decrease seen in the FSHβ and the 39% decrease seen in the ActRIIA knockout mice. Comparing the functional activity of the Sertoli cell in terms of its germ cell-carrying capacity, the ratio of round spermatids to Sertoli cells in the double knockouts was 4.93 ± 0.2 compared with 9.7 ± 0.6, 5.44 ± 0.21, and 6.94 ± 0.47 in the controls, FSHβ mutants, and ActRIIA knockouts, respectively. Similarly, there were substantial reductions in all germ cell types from spermatogonia to elongated spermatids in single and double knockouts (Fig. 3) (34) compared with WT controls. The reduction in type A and I spermatogonia was particularly marked (Fig. 3). The numbers of preleptotene and B spermatogonia were less marked in their reduction compared with type A and I spermatogonia. More advanced germ cell types showed a gradual attrition in numbers compared with wild-type mice. The ratio of round spermatids to pachytene spermatocytes was not different in the knockout and wild-type mice. These data suggest that despite the absence of both FSH and ActRIIA, the double mutants demonstrate only a moderate effect on testis cell populations and a significant reduction in type A and I spermatogonia.

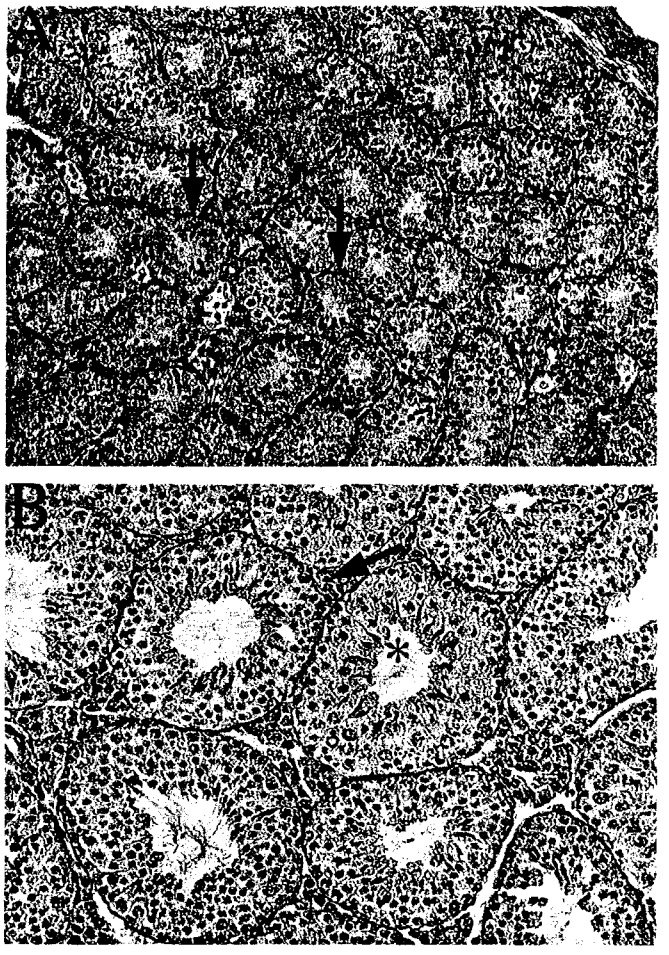


FIG. 2. Histology of the testis from FAR double mutant male mice. Testis from 2-wk-old (A) or 6-wk-old (B) double mutant mice was fixed in Bouin's reagent. Paraffin-embedded 5-μm sections were cut and stained with PAS reagent. The seminiferous tubules appear normal in A (*arrows*), and in B, a normal Leydig cell is indicated. Grossly normal stages of spermatogenesis are present in the tubules in B, including many late stage spermatids. A seminiferous tubule with sperm tails in the lumen is indicated with an *asterisk*. Although histology of the testes is normal, the FAR double mutant mice have reduced epididymal sperm counts at 6 wk of age, and these male mice sire a reduced number of pups when mated to double heterozygous female mice.

TABLE 2. Intratesticular testosterone content in wild-type and mutant mice

Genotype		Testosterone (ng/mg testis)	No. of mice
FSH	ActRIIA		
+/+	+/+	0.1 ± 0.04^a	5
−/−	+/+	0.7 ± 0.3^b	5
+/+	−/−	0.6 ± 0.2^c	6
−/−	−/−	0.7 ± 0.2^d	5

Adult male mice at 42 d were used. Testis extracts were prepared according to the methods described (29). The intratesticular content was quantitated by a solid phase RIA. Values are the mean ± SEM. By *t* test: *a vs. b* or *c* or *d*, $P < 0.05$; by single factor ANOVA, *b vs. c* or *d* and *c vs. d*, $P > 0.05$. +/+, Wild-type; −/−, homozygous mutant.

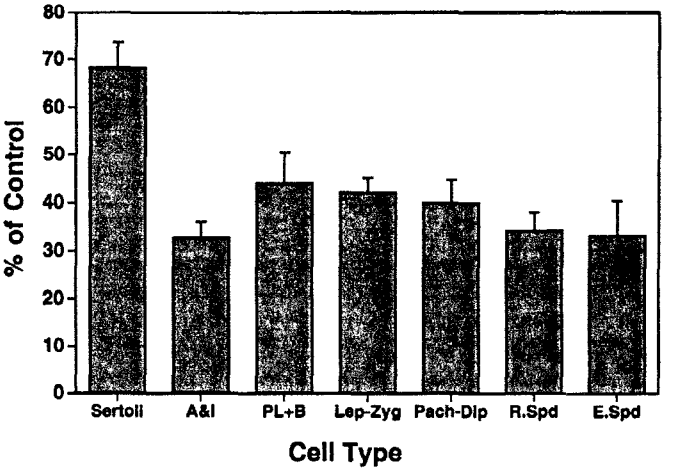


FIG. 3. Stereological analysis of cell types in the testis of FAR double mutant mice. The testis samples from wild-type and FAR double mutant mice were sectioned and stereologically analyzed. The numbers of different cell types in the double mutant testis are represented as a percentage of the control by comparing the values obtained with wild-type control testis samples. The only significant difference is seen with type A and type I spermatogonia, which are further decreased compared with single mutants. The numbers of other cell types are comparable to those in the single mutants.

Testicular gene expression profiles are unaltered in FAR double mutant mice

The coordinated expression of several genes at distinct stages of spermatogenesis is critical for normal male fertility. To analyze the gene expression profiles of various cell-specific markers in the testis, we performed Northern blot analysis on total RNA prepared from adult (42-d-old) wild-type, ActRIIA-null, and FAR double mutant mouse testes and hybridized with specific radiolabeled cDNA probes for these genes. Densitometric quantitation of the Northern blot data in Fig. 4 showed that there were no significant changes ($P > 0.05$, by single factor ANOVA) in the expression profiles of cell-specific markers such as inhibin α (Sertoli cell-specific), LH receptor (Leydig cell-specific), protamine 1 (spermatid-specific), and germ cell nuclear factor (germ cell-specific) among the three groups. However, the significant differences in expression were only in protamine 2 [spermatid-specific; 2.6 (wild-type), 2.3 (ActRII$^{-/-}$) *vs.* 1.6 (FAR double mutant); $P < 0.05$, by single factor ANOVA] and CREM (spermatocyte-specific; 2.5 (wild-type), 2.2 (ActRII$^{-/-}$) *vs.* 1.1 (FAR double mutant); $P < 0.05$, by single factor ANOVA] expression when ActRIIA null and WT groups were compared with FAR mutants. To further verify the possibility that the unaltered expression of most of these genes was due to a compensatory increase in ActRIIB, the other activin type II receptor, we also hybridized the Northern blot with an ActRIIB-specific cDNA probe (Fig. 4, *left, top panel*). Although ActRIIB appeared to be reduced in the testis of ActRIIA-null mice compared with that in WT testis, the absence of both FSH and ActRIIA did not result in any compensatory increase in ActRIIB mRNA expression [0.8 (WT), 0.8 (ActRII$^{-/-}$), 0.7 (FAR double mutant); $P > 0.05$, by single factor ANOVA] in the testis of double mutant mice. Hence, these expression data indicate that the male

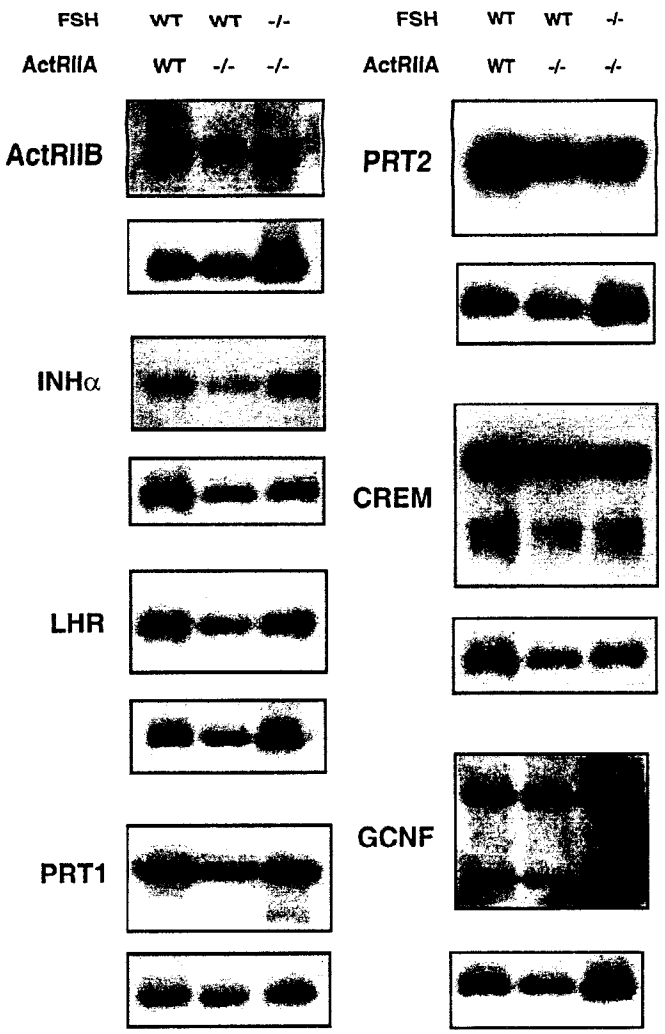


FIG. 4. Analysis of gene expression in WT, FSHβ knockout, and FAR double mutant testes. Twenty micrograms of total RNA from testes of 42-d-old male mice were separated on agarose gels, transferred to nylon membranes, and hybridized with cDNA or genomic probes corresponding to ActRIIB, inhibin α (INH α), LH receptor (LHR), protamines 1 and 2 (PRT1, PRT2), cAMP-responsive element modulator (CREM), and germ cell nuclear factor (GCNF). In each case, the blots were stripped and reprobed with an 18S RNA probe to confirm equal loading of RNA samples in each lane. The densitometric data were analyzed by single factor ANOVA. Significant differences in the gene expression profiles of only PRT-1 and CREM, but not other testis cell-specific markers, are seen in the three groups analyzed.

reproductive phenotypes in the absence of FSH and ActRIIA signaling are not due to compensatory mechanisms involving up-regulation of ActRIIB or other marker gene-encoded mRNA expression in the testis of FAR double mutant mice.

Apoptosis as detected by terminal transferase-mediated deoxy-UTP nick end labeling staining is similar in the FAR double mutant mice compared with single mutants

Apoptosis in WT mice generally occurs in clusters (three to five cells) localized to a subset (~20%) of the seminiferous tubules (data not shown). A similar pattern was observed in the double mutants. In both groups the cells affected tended to be in the layers corresponding to spermatogonia and primary spermatocytes (data not shown).

Discussion

To analyze the local role of ActRIIA signaling *in vivo* in the testis independent of FSH, we produced double mutant mice lacking both FSH and ActRIIA (FAR mice) by a genetic intercross. Although several mouse models with mutations in activin ligands, their cognate receptors, and binding proteins were produced previously by our group and others, these are not suitable for analyzing the local role of activins *in vivo* within the testis. For example, activin A deficiency in mice results in perinatal lethality due to multiple craniofacial defects (35), and only female, not male, mice lacking activin B demonstrate reproductive defects (36). Mice in which activin β_B is expressed from the activin β_A locus using a knock-in strategy also have male reproductive defects in addition to a partial rescue of other phenotypes of activin A-deficient mice (12). ActRIIB and activin receptor type IA, IB-null mice have craniofacial, cardiovascular, and embryonic turning defects, and all die embryonically or perinatally (37–39). Finally, follistatin (activin-binding protein)-null mice have skin and sternal defects and die perinatally (40). Tissue-specific knockout mouse models with deletion of activins or any of the activin signaling pathway components are not yet available. ActRIIA deficiency leads to embryonic lethality, but in only a minor proportion of mutant embryos (~22%); all of the viable mice have suppressed FSH levels and demonstrate reproductive defects (23). Hence, FAR double mutant mice are an important model to examine the local effects of activins in the testis in the absence of both ActRIIA signaling and FSH.

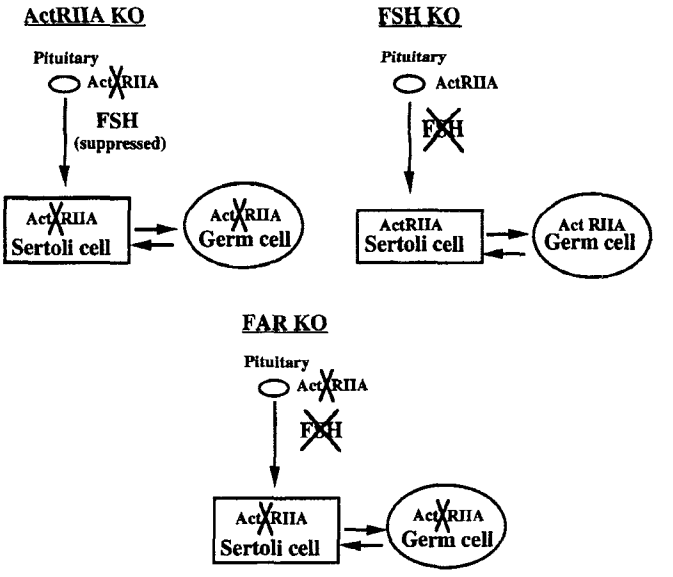
Male fertility is not impaired in single mutant mice lacking either FSH or ActRIIA. Similarly, FAR double mutant mice are fertile despite the absence of both FSH and ActRIIA; however, their fertility is greatly reduced. This reduced fertility in FAR double mutant mice may be explained by reduced epididymal sperm number compared with that in mice lacking either FSH or ActRIIA alone. The absence of a direct effect of ActRIIA signaling within the epididymis (in addition to the indirect effects of FSH) in double mutant mice may result in significantly reduced epididymal sperm counts. There is evidence to demonstrate that in the rat epididymis, mRNAs encoding α, β_A, and β_B subunits are expressed, and these observations would support a direct role of ActRIIA signaling within the epididymis (de Kretser, D., unpublished data). The absence of any noticeable defect in the testis and the presence of qualitatively normal spermatogenesis in the absence of both FSH and ActRIIA (in the double mutants) suggest that a variety of other growth factor signaling pathways are important for testis development and function.

Stereological data indicate a decrease in type A and I spermatogonia, which is the only noticeable defect in the testis of FAR double mutants compared with the single mutants (34). The comparison is consistent with the proposition that the double knockout has some additive effects, although the final resolution of this issue requires an FSH replacement

study. Despite the marked reduction in germ cell production, expression profiles of important marker genes are unaltered in the testis of FAR double mutant mice. The data from Northern blot analysis support our hypothesis that ActRIIB, the other activin type II receptor (not up-regulated in the absence of ActRIIA), whose expression was barely detectable by *in situ* hybridization (data not shown), cannot compensate for ActRIIA function in the testis. We cannot, however, rule out the possibility that the absence of FSH and ActRIIA leads to changes in gene expression profiles of other testis-specific genes not analyzed in the present study. Those genes may be downstream of ActRIIA signaling and are stage-specifically expressed during spermatogenesis. Within the testis, ActRIIA mRNA and protein are localized to Sertoli cells, pachytene spermatocytes (21, 22), and round spermatids. The signal transducer, Smad2, which is implicated downstream of ActRIIA signaling, has also been shown to be coexpressed in these testis cell types (41), suggesting the existence of a full complement of activin-signaling components in these cells. It will be important to analyze in the future the alterations in this signaling pathway in FAR double mutants.

Our genetic analysis with FAR double mutant mice confirms an important local role of ActRIIA signaling (in the testis) independent of its effects on FSH homeostasis. Based on our analyses with mice lacking FSH, ActRIIA (34), and both FSH/ActRIIA, a hypothetical model can be formulated, as shown in Fig. 5. In ActRIIA-null mice, FSH levels are suppressed, leading to a reduction in Sertoli cell number accompanied by reduced germ cell and sperm number. In this model the direct effects of ActRIIA signaling within spermatogenic cells are masked by the FSH-mediated effects secondary to the absence of ActRIIA signaling. This is further confirmed by a comparable reduction in Sertoli cells, germ cells, and sperm number in FSH-null mice, in which ActRIIA signaling is intact. The local effects of ActRIIA signaling directly on the spermatogenic cells are readily apparent in the FAR double mutant mice (absence of both FSH and ActRIIA), which have a further reduction in type A and I spermatogonia and sperm, but not in Sertoli cell number. More quantitative analyses and mechanistic studies are required to clearly establish this model supporting the local role of ActRIIA *in vivo*.

In summary, male mutant mice lacking both FSH and ActRIIA demonstrate reduced number of progeny, reduced epididymal sperm counts, and reduced type A and I spermatogonia, but no significant changes in many of the testis gene expression profiles and Sertoli cell number compared with mice lacking either FSH or ActRIIA alone. Future studies will involve exploring the molecular mechanisms of ActRIIA signaling through the SMAD pathway directly in the spermatogenic cells. It will be also important in the future to genetically abolish ActRIIA and ActRIIB signaling *in vivo* selectively in the testis by a Cre-lox recombination technology (42). Such a strategy will be useful to analyze the testicular phenotypes directly in the absence of activin signaling only in the testis, without affecting activin signaling in pituitary FSH homeostasis.



Genotype	Sertoli cells	Germ cells
Wild-type	100	100
ActRIIA KO	39	47
FSH KO	30	47
FAR KO	32	32

FIG. 5. Model to explain the testicular phenotypes in ActRIIA knockout, FSH knockout, and FAR double knockout (FAR KO) mice. The testis phenotypes of both ActRIIA KO and FSH KO mice are almost identical, suggesting that ActRIIA signaling is important for FSH homeostasis and consequently for Sertoli/germ cell function. The dramatic reduction in epididymal sperm counts and a considerable drop in type A and I germ cell populations in FAR knockout mice confirm that ActRIIA signaling is important locally within the testis, independent of its effects on FSH homeostasis. The approximate percent reductions in Sertoli cells and germ cells in the different groups are indicated at the *bottom*.

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Address all correspondence and requests for reprints to: Dr. T. Rajendra Kumar, Department of Pathology, One Baylor Plaza, Baylor College of Medicine, Houston, Texas 77030. E-mail: tkumar@bcm.tmc.edu.

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