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June 23, 2003

Dr. Richard N. Sifers
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Dear Dr. Sifers:

Enclosed please find the Annual Progress report of The Moran Foundation project entitled " REGULATION OF SERTOLI CELL PROLIFERATION IN THE MOUSE TESTIS". The project was initiated in November 2002 and we have made significant progress with all the experiments proposed. Four manuscripts have been published during this one year period and two manuscripts acknowledge the financial support from The Moran Foundation. Two more manuscripts based on the proposed studies are in preparation and will soon be submitted.

I thank The Moran Foundation for their generous support of our research work.

Sincerely,

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Annual Report of the project:

" REGULATION OF SERTOLI CELL PROLIFERATION IN THE MOUSE TESTIS"

November 01, 2002-June 30, 2003

T. Rajendra Kumar, Ph.D.
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Progress Report

The goal of the project was to characterize the role of p27 in Sertoli cell proliferation and differentiation. We have successfully accomplished this goal by generating transgenic mice that expresses p27 only in the Sertoli cell lineage of the testis. Since the submission of the project, 3 additional founders (2 female and one male) were generated. While both the female founders transmitted the transgene to F-1 generation, the male founder was infertile. Testes protein extracts were prepared from the F-1 male progeny, and immunoblot analysis was performed with p27 and PCNA antibodies. The results demonstrate an inverse correlation to PCNA and p27 expression. This difference is clearly apparent in testes protein extracts from transgenic lines. During the course of these studies we have also uncovered the mechanism of thyroid hormone induced Sertoli cell proliferation in the mouse testis. Our studies demonstrate that hypothyroidism leads to a marked decrease in p27 levels resulting in enhanced proliferation of Sertoli cells. We are beginning to understand how both positive and negative regulators affect Sertoli cell proliferation.

We believe that our further analyses will establish a number of genes/proteins involved downstream of these regulators in the Sertoli cells. We will eventually understand how they will interact together and orchestrate spermatogenesis during testis development. **Clearly, the studies initiated in this project through the support from The Moran Foundation have immense potential and form the basis for all our future investigations of this project.**

Publications (* The Moran Foundation Support acknowledged)

1) Baker PJ, Pakarinen P, Huhtaniemi IT, Abel, MH, Charlton HM, **Kumar TR**, and Shaugnessy PO. (2003) Failure of normal Leydig cell development in FSH receptor-deficient mice but not FSHb-deficient mice-role for constitutive receptor expression. **Endocrinology** 44:138-40.

2) Abel MH, Huhtaniemi IT, Pakarinen P, **Kumar TR** and Charlton HM. (2003) Age related uterine and ovarian hypertrophy in follicle stimulating hormone (FSH) receptor knockout and FSHβ subunit knockout mice. **Reproduction**125: 165-173.

3) Céline Lukas-Croisier, Celina Lasala, Juliette Nicaud, Patricia Bedecarrás, **Kumar T.R.,** Martin Dutertre, Martin M. Matzuk, Jean-Yves Picard, Nathalie Josso, Rodolfo Rey. (2003) Follicle stimulating hormone increases testicular anti-Müllerian hormone (AMH) production through Sertoli cell proliferation and cyclic AMP-mediated CRE-independent activation of the AMH gene. **Molecular Endocrinology** 17: 550-561. *

4) Matzuk MM, Hadsell, LA, De Mayo F and **Kumar TR.** (2003) Overexpression of human chorionic gonadotropin in transgenic mice causes multiple reproductive defects. **Biology of Reproduction**, 338-346. *

5) Dass B and **Kumar TR** (2003) Regulation of G1-S phase cell cycle regulators in the testis of FSH-knockout mice. (**Manuscript in Preparation**)

6) Huang S, and **Kumar TR** (2003) Germ cell independent differentiation of Sertoli cells in the atrichosis (*at*) mutant mouse. (**Manuscript in Preparation**)

Invited Seminars:

1) Institute of Reproduction and Development, Imperial College, London. (May 2003)
2) National Endocrinology and Nutrition Symposium, Caceres, Spain. (May 2003)

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Failure of Normal Leydig Cell Development in Follicle-Stimulating Hormone (FSH) Receptor-Deficient Mice, But Not FSHβ-Deficient Mice: Role for Constitutive FSH Receptor Activity

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Previous studies have suggested that FSH may be involved in regulation of Leydig cell function. We have examined this directly using two mouse models with null mutations in either the FSH β-subunit (FSHβKO mice) or the FSH receptor (FSHRKO mice). Circulating LH levels were normal in adult FSHβKO mice, but were significantly increased in FSHRKO mice. Intratesticular testosterone levels increased normally in FSHβKO mice from birth to adulthood, whereas testosterone levels in FSHRKO mice failed to increase normally after puberty and were significantly reduced in adult animals. This was associated with reduced levels of mRNA encoding cytochrome P450 side-chain cleavage, 3β-hydroxysteroid dehydrogenase type VI, and steroidogenic acute regulatory protein in FSHRKO mice. Leydig cell number was normal in FSHβKO mice during development, but in FSHRKO mice Leydig cell number increased slowly after puberty and was significantly reduced in the adult animal. Transfection studies showed that the FSHR exhibits constitutive activity in the absence of agonist stimulation. The results indicate, therefore, that Sertoli cells regulate the development of Leydig cell number and that constitutive activity within the FSHR is sufficient to stimulate this process. The presence of the hormone itself is not required when circulating LH levels are adequate. (*Endocrinology* 144: 138–145, 2003)

ANDROGEN PRODUCTION and fertility in the adult male are dependent upon Leydig cell activity in the testis. These cells, in turn, depend crucially on LH secreted from the pituitary in response to GnRH. This is clearly illustrated by the failure of postnatal androgen production in mice lacking either GnRH peptide or the LH receptor (1–3). There is, in addition, a body of evidence suggesting that Leydig cell growth, activity, and survival are dependent upon the Sertoli cell population of the testis. This hypothesis dates back to earlier studies showing that FSH can act to increase Leydig cell activity in hypophysectomized rats or GnRH-deficient (*hpg*) mice (4–11). As FSH receptors (FSHR) are present only in the Sertoli cells in the testis, this has been taken as evidence of Sertoli cell regulation of Leydig cell function. These earlier studies on the normal role of FSH in regulating the Leydig cell population were complicated by two issues. Firstly, circulating LH was markedly reduced or absent in the models used, and the Leydig cells were lacking any normal trophic stimulation. Secondly, in most cases [with two exceptions (9, 11)] the studies predated the introduction of recombinant FSH, and the FSH preparations used contained low, contaminating levels of LH. It is clear from more recent studies, in which Sertoli cell apoptosis was shown to be followed by Leydig cell degeneration (12), that there is a clear link between the two cell types, but the normal role of FSH in regulating Leydig cell development and activity remains unclear.

The recent generation of different models of FSH deficiency in the mouse now allows us to address this issue of FSH involvement in Leydig cell development and function. Mice lacking FSH through a null mutation in the β-subunit (FSHβKO mice) were first described by Kumar *et al.* (13) and were shown to be fertile, but with reduced testis size. Later, two separate laboratories described the development of an FSHR-null (FSHRKO) mouse that is also fertile with reduced testis size (14, 15). Interestingly, circulating testosterone levels are reported to be normal in adult FSHβKO mice (13), but to be reduced in FSHRKO mice (14). In this study we examined Leydig cell development and function in both models directly. The results show that Leydig cell number and function appear normal in FSHβKO mice, but that Leydig cell number and, hence, androgen production are reduced in FSHRKO mice. Further studies indicate that this difference may be due to the presence of constitutive activity within the FSHR in the absence of hormone ligand.

Abbreviations: FSHR, FSH receptor; FSHRKO, FSH receptor knockout; hCG, human chorionic gonadotropin; 3β-HSD IV, 3β-hydroxysteroid dehydrogenase type VI; mFSHR, mouse FSH receptor; P450scc, cytochrome P450 side-chain cleavage; StAR, steriodogenic acute regulatory protein.

Materials and Methods

Animals

Generation of both FSHβKO and FSHRKO mice has been described previously (13, 15). Both colonies were raised on a C57BL6 background and were maintained at University of Oxford under United Kingdom

Home Office regulations. As both FSHRKO and FSHβKO adult males are fertile, the colonies were maintained by breeding homozygous males with heterozygous females. Heterozygous males were used as the control group in this study. Animals were killed at 1, 5, 20, 60, 120, and 180 d of age (day of birth is d 1). One testis from each animal was frozen in liquid N_2, and the other testis was fixed overnight in Bouin's fluid before being stored in 70% ethanol. Blood was also collected from adult animals, and serum was stored frozen at −20 C before measurement of LH. To measure the responsiveness of testes *in vivo* to exogenous stimulation, adult animals (60 and 180 d old) were injected ip with human chorionic gonadotropin (hCG; 0.5 IU/g; Serono Pharmaceuticals Ltd., Feltham, UK) in saline. Animals were killed 1 h after injection, and testes were stored frozen at −20 C until extracted for hormone assay.

Hormone and second messenger assays

To measure intratesticular testosterone levels steroids were extracted from the testes in ethanol and measured by RIA as previously described (16). Levels of progesterone in culture medium were measured directly by RIA (17). Serum levels of LH were measured using an in-house immunofluorometric assay (Delfia, Wallac, Inc., Turku, Finland) as previously described (18). Levels of cAMP in culture medium were measured by RIA (19).

Stereology and histology

For stereology, testes were fixed in Bouin's solution and stored in 70% ethanol. Tissue was embedded in Technovit 7100 resin (TAAB Laboratories Ltd., Reading, UK), cut into sections (20-μm thickness), and stained with Harris' hematoxylin. The total testis volume was estimated using the Cavalieri principle (20), and the slides used to estimate the number of cells were also used to estimate testis volume. The optical disector technique (21) was used to count the number of Leydig cells in each testis. The numerical density of Leydig cells was estimated using an Olympus BX50 microscope fitted with a motorized stage (Prior Scientific Instruments, Cambridge, UK) and Stereologer software (Systems Planning Analysis, Alexandria, VA).

To generate semithin (1-μm) sections, testes were fixed in 1% glutaraldehyde-4% paraformaldehyde in phosphate buffer (0.1 M), pH 7.2, for 24 h at 4 C and embedded in araldite. Cut sections were stained with toluidine blue.

Measurement of mRNA levels

Real-time PCR was used to measure levels of specific mRNA species present in the testes of control, FSHRKO, and FSHβKO animals (22). To allow results to be compared directly on a per testis basis, a standard amount of external control mRNA (luciferase) was added to each sample during the RNA extraction phase (22, 23). Sequences of primers and probes and the reaction conditions used were previously described (23).

Cell transfections

A mouse Leydig tumor cell line, mLTC-1 (24), was cultured in HEPES-buffered Waymouth's medium (Sigma, St. Louis, MO) supplemented with 9% heat-inactivated horse serum (Life Technologies, Inc., Paisley, UK), 4.5% heat-inactivated fetal calf serum (BioClear, Calne, UK), and 50 mg/liter gentamicin (Biological Industries, Hemeek, Israel) at 37 C in 5% CO_2. Cells were transiently transfected with an expression vector containing the mouse FSHR (pSG5-mFSHR-WT), the mouse FSHR with an inactivating point mutation (pSG5-mFSHR-C566T) (25, 26), or vector alone (pSG5) using Lipofectamine reagent (Life Technologies, Inc., Paisley, UK). Transfection efficiencies were verified by co-transfections with a luciferase expression vector. For transfections, cells were cultured on 10-cm diameter tissue culture plates to confluence of 50–70%, and 9 μg pSG construct vector or 0.3–0.6 μg luciferase vector were used to transfect the cells in Opti-MEM I with Glutamax (Invitrogen, Paisley, UK). After 5 h an equal volume of Waymouth's medium supplemented with twice the normal concentration of serum was added to the cells. One day after the transfection cells were trypsinized and counted, and 60,000 cells/well were plated on 24-well plates. After 24 h cells were washed, and 1 ml Opti-MEM I with Glutamax plus 100 nmol/liter 1-methyl-3-isobutylxanthine (Alcon Laboratories, Inc., Milwaukee, WI) were added per well. Cells were incubated at 37 C in quadruplicate under basal conditions or in the presence of recombinant human FSH (National Hormone and Pituitary Program, McKesson BioServices, Rockville, MD). Aliquots of the incubation medium were collected after 2 h for cAMP and after 6 h for progesterone measurements.

Statistical analysis

Differences between control, FSHRKO, and FSHβKO animals were initially analyzed by single or two-factor ANOVA. Where a significant overall difference between animal groups was detected, differences between individual means were assessed by the Newman-Keuls test (after single factor analysis) or by *t* tests using the pooled variance (after two-factor analysis).

Results

Intratesticular testosterone levels

In control animals from both FSHβKO and FSHRKO groups testosterone levels per testis were low from birth to d 20, then increased markedly to d 60 and continued to rise up to d 180 (Fig. 1). In both FSHβKO and FSHRKO animals testosterone levels were normal in the prepubertal period up to d 20. After d 20 testosterone levels increased normally in the FSHβKO group, and on d 180 there was no significant difference from control values. In contrast, testosterone levels in FSHRKO mice increased more slowly after d 20 and were significantly less than control values on d 180 (Fig. 1).

To measure the responsiveness of testes from FSHβKO and FSHRKO groups to exogenous stimulation by hCG, adult animals were injected with hCG as described in *Materials and Methods*. After hCG treatment, there was an increase in intratesticular testosterone levels in all groups, but levels in both FSHβKO and FSHRKO animals were significantly reduced compared with control values (Fig. 2). No difference was found between the two knockout models in their response to hCG.

Circulating LH levels

Levels of circulating LH in adult FSHβKO mice did not differ significantly from those in control animals (Fig. 3). In contrast, LH levels in FSHRKO mice were significantly increased compared with control values (Fig. 3).

Expression of Leydig cell mRNA levels

Levels of mRNA encoding the steroidogenic enzymes cytochrome P450 side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase type VI (3βHSD VI), cytochrome P450 17α-hydroxylase (P450c17), and 17βHSD III and the steriodogenic acute regulatory protein (StAR) were measured by real-time PCR. Levels of mRNA were measured relative to an externally added standard (luciferase) that allows direct comparison of mRNA levels between groups (22). For each mRNA species, mean levels in FSHRKO mice were lower than those in control animals, and this difference was significant for P450scc, 3βHSD VI, and StAR (Fig. 4). In FSHβKO mice expression levels of the mRNA species measured were either normal (P450scc, 3βHSD VI, and P450c17) or slightly, but significantly, increased (17βHSD III and StAR).

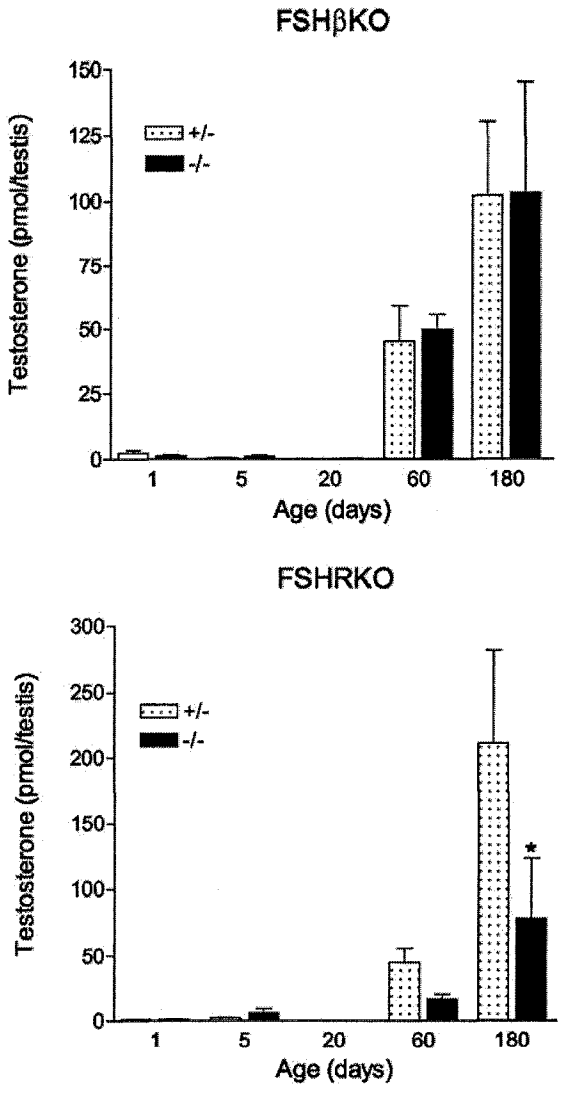

FIG. 1. Intratesticular testosterone concentrations in control heterozygous animals (+/−) and in FSHβKO (−/−) and FSHRKO (−/−) animals during postnatal development. The results show testosterone levels per whole testis and represent the mean ± SEM of 4–17 animals/group. *, Significant ($P < 0.05$) difference from age-matched controls.

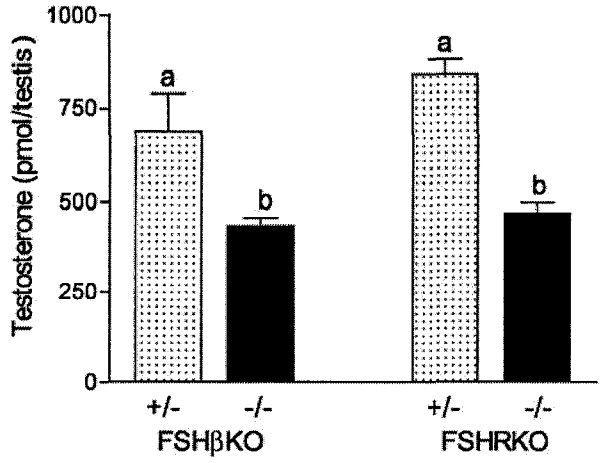

FIG. 2. Intratesticular testosterone levels per testis in adult control heterozygous mice (+/−) and in adult FSHβKO (−/−) and FSHRKO (−/−) mice after hCG injection. Animals were injected with hCG (0.5 IU/g) and were killed 1 h later. The mean ± SEM of four to nine animals per group are shown. Both 60- and 180-d-old animals were included in each group, but there was no age-dependent difference in testicular testosterone content after hCG injection, and data from different aged animals were pooled. Groups with *different letter superscripts* are significantly ($P < 0.05$) different.

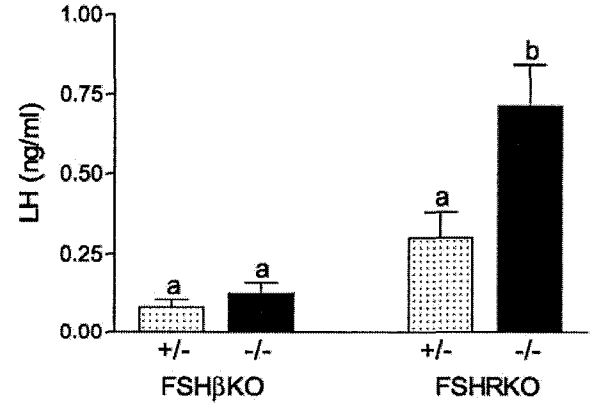

FIG. 3. Circulating LH levels in adult control heterozygous mice (+/−) and in adult FSHβKO (−/−) and FSHRKO (−/−) mice. The mean ± SEM of 4–12 animals/group are shown. The animals used in this study were 60 d old. Groups with *different letter superscripts* are significantly ($P < 0.05$) different.

Testis size, Leydig cell number, and interstitial morphology

Testis size was reduced to about one third of normal in both FSHβKO and FSHRKO mice with the effect evident after d 5 (Fig. 5). Leydig cell number in both FSHβKO and FSHRKO mice was normal at birth and up to d 5 postnatally (Fig. 6). After d 5 Leydig cell number increased markedly in control animals, reaching a peak in adult animals. In FSHβKO mice Leydig cell development was normal after d 5, and numbers in the adult animal were not significantly different from control values. In contrast, Leydig cell numbers in FSHRKO mice failed to increase normally after d 5 and were significantly less than control values in the adult animal (Fig. 6). Despite differences in Leydig cell number interstitial cell morphology was similar in control, FSHβKO, and FSHRKO mice at the light microscopic level (Fig. 7).

Constitutive FSHR activity

To determine whether the FSHR shows constitutive activity, a Leydig cell line was transfected with either wild-type FSHR (mFSH-WT) or control vector, and the activity of the cells was measured. Monitoring by luciferase coexpression showed that transfection efficiencies with the different plasmids were similar in the individual experiments. The results in Fig. 8A show data from a single experiment in which cAMP levels were measured after transfection, and it is clear that there was a significant increase in basal cAMP production in the presence of the receptor and the absence of ligand. In contrast, transfection with an FSHR containing an inactivating point mutation (mFSH-C566T) had no effect on basal expression. As expected, transfection with mFSHR also conferred sensitivity to FSH, which was significantly reduced in cells transfected with mFSH-C566T. Figure 8B shows the

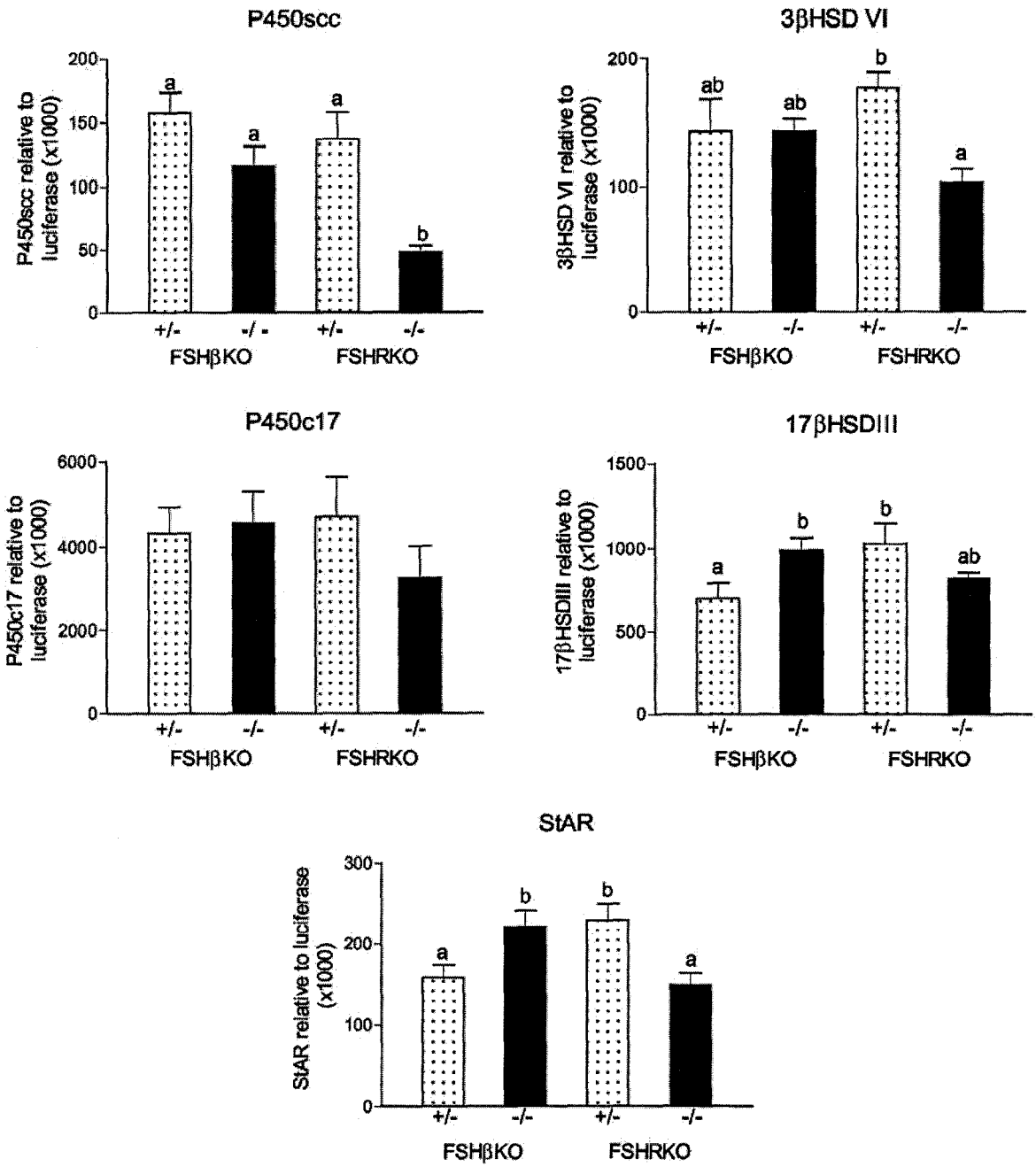

FIG. 4. Measurement of Leydig cell-specific mRNA species by real-time PCR. The results show expression in control heterozygous mice (+/−) and in FSHβKO (−/−) and FSHRKO (−/−) mice. RNA was extracted from whole testes of adult animals and reverse transcribed to generate cDNA. Levels of specific mRNA species were measured by real-time PCR and expressed relative to an external control (luciferase). As the same amount of luciferase mRNA was added to each sample, the results can be compared directly. Results show the mean ± SEM of four animals in each group. The animals used in this study were 180 d old. Groups with *different letter superscripts* are significantly ($P < 0.05$) different.

combined results from five experiments designed to measure changes in basal cAMP and progesterone production by Leydig cells after transfection with the FSHR. Overall, there was an approximately 4-fold increase in both measures of basal cell activity after transfection with the receptor.

Discussion

FSH is a member of the heterodimeric glycoprotein hormone family, which includes LH, hCG, and TSH. Members of this family share a common α-subunit with biological specificity conferred by a hormone-specific β-subunit, although only the heterodimers show biological activity. As with other members of the family, FSH acts through a single receptor type although considerable variation in the receptor can be induced through alternate splicing (26, 27). It might be expected, therefore, that the effects of induced null mutations in the hormone β-subunit gene or the hormone receptor gene would induce similar phenotypes in the affected

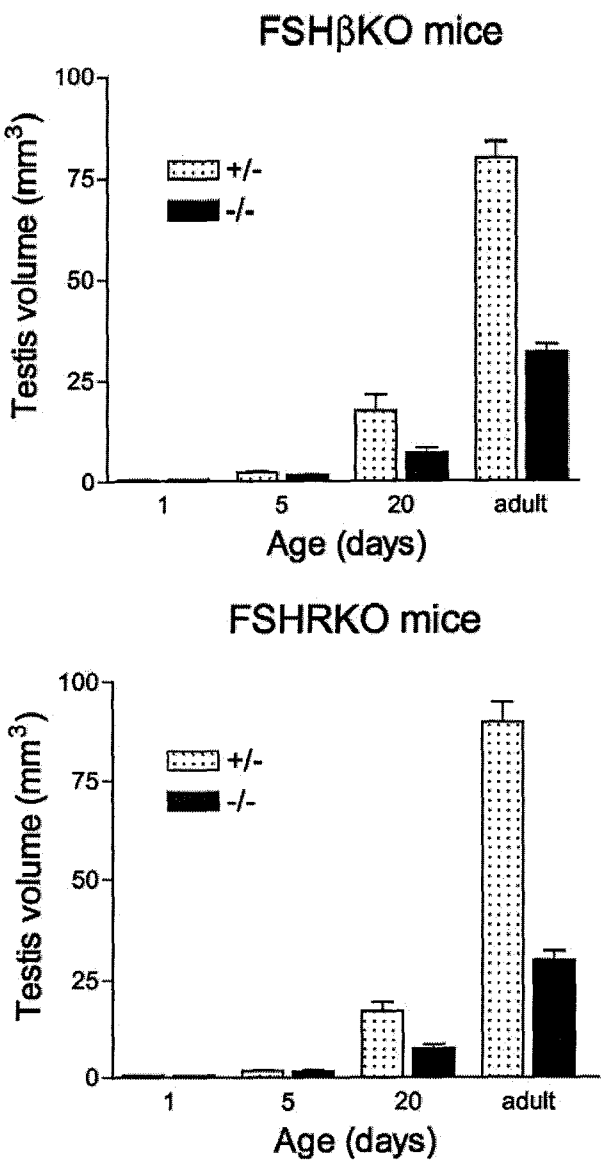

FIG. 5. Changes in testicular volume during postnatal development in control heterozygous (+/−) mice and in FSHβKO (−/−) and FSHRKO (−/−) mice. Results show the mean ± SEM of three to five animals in each group.

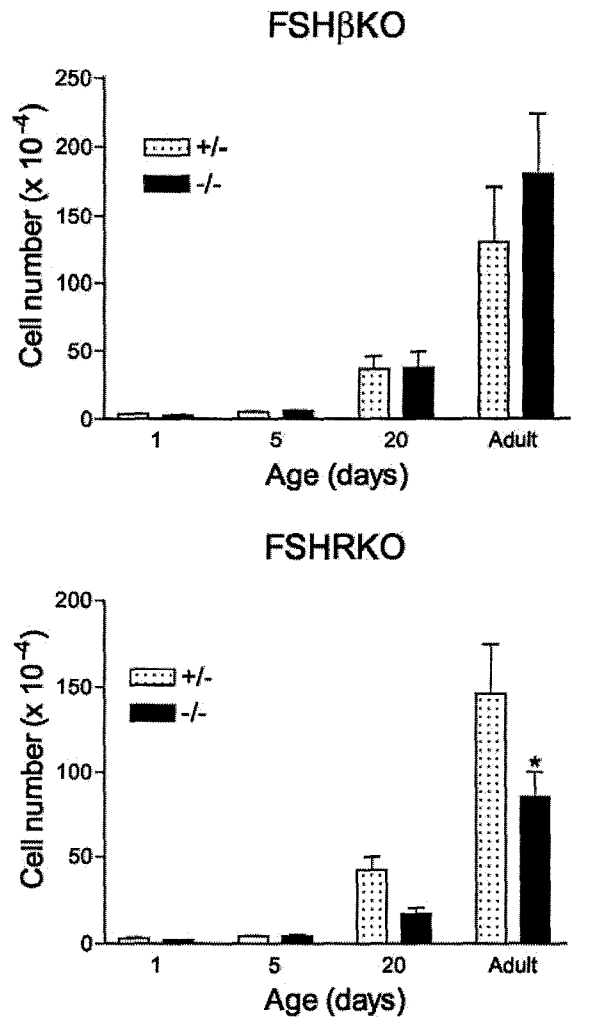

FIG. 6. Leydig cell number during development in control heterozygous mice (+/−) and in FSHβKO (−/−) and FSHRKO (−/−) mice. Leydig cell number was measured using the optical dissector method. Results show the mean ± SEM of three to five animals in each group. *, Significant ($P < 0.05$) difference from age-matched controls.

animals. Consistent with this, it has been shown that both FSHβKO and FSHRKO male mice are fertile, but have reduced testis size and reduced levels of spermatogenesis (13–15). The results reported here, however, show that there are significant differences in the effects of the mutations on Leydig cell development in affected animals.

Leydig cell development appears to be normal in FSHβKO mice, with no difference in Leydig cell number during development or in the adult animal and no difference in testosterone or circulating LH levels. This confirms previous reports showing that Leydig cell number and circulating testosterone levels are normal in adult FSHβKO mice (28). In contrast, testosterone production by FSHRKO mice was reduced despite an increase in circulating LH, and this was associated with reduced testicular expression of key mRNA species associated with steroidogenesis in Leydig cells. It is likely, however, that these apparent changes in Leydig cell function are due to a failure of normal Leydig cell proliferation/differentiation during puberty in FSHRKO mice, causing the numbers of cells in the adult animal to be reduced to about 60% of normal. This would explain the reduction in levels of intratesticular testosterone and Leydig cell-specific mRNA species and the rise in circulating LH, which is inversely linked to overall changes in testosterone production. In a recent study of another FSHRKO mouse model, testosterone levels were also significantly reduced in the adult animals (29), and it is likely that this is due to the same mechanism of action.

When a more marked phenotype arises from a receptor null mutation compared with a hormone null mutation, it is likely that this is either because the receptor is not specific to the hormone targeted with the mutation or because the receptor itself shows constitutive activity in the absence of agonist stimulation. In the case of the FSHR, there is as yet no evidence that a growth factor or hormone, other than FSH,

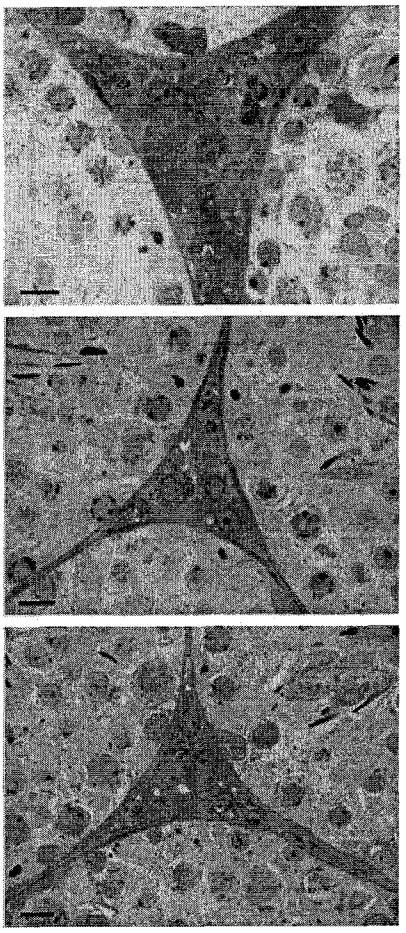

FIG. 7. Light micrographs showing interstitial tissue in control, FSHβKO, and FSHRKO mice. The animals used to prepare these micrographs were 60 d old. *Bar*, 10 μm. The control animal used in this montage was an FSHβ heterozygote. There were no clear differences in the morphology of the interstitial tissue between the different groups of animals at the light microscopic level.

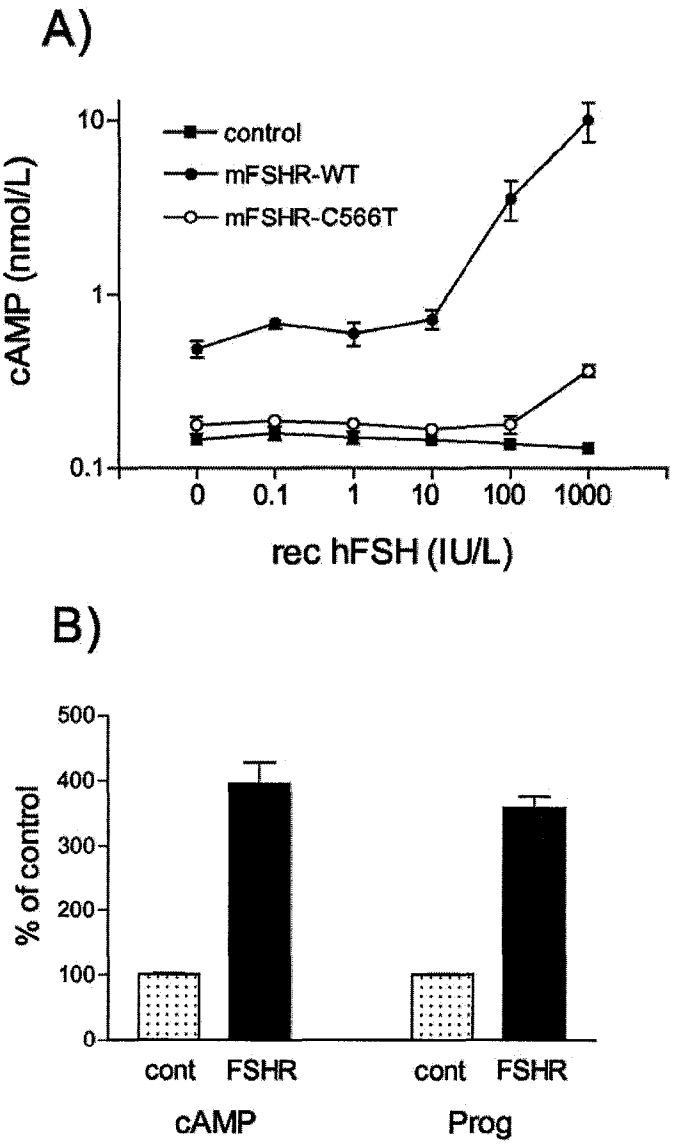

FIG. 8. Progesterone and cAMP production in a Leydig cell line transfected with mFSHR (mFSHR-WT), mFSHR with an inactivating point mutation (mFSHR-C566T), or vector only (control). A, Results from a single experiment showing cAMP production by transfected cells under basal conditions and in the presence of increasing levels of hFSH. Results show the mean ± SEM of quadruplicate wells. B, Combined data from five experiments showing basal cAMP and progesterone production in cells transfected with mFSHR-WT or vector alone (control). Results show the mean ± SEM.

can activate the receptor. Previous studies have shown, however, that G protein-coupled receptors can display constitutive activity (30), and studies reported here show clearly that the FSHR will express constitutive activity in the absence of ligand. The FSHR normally shows down-regulation in the presence of FSH (31), and it would be expected, therefore, that receptor levels will be increased in FSHβKO mice. As constitutive activity varies directly with receptor number, this will serve to increase activation of the cells and provides a clear explanation for phenotypic differences between FSHRKO and FSHβKO mice. In this respect the FSH and TSH receptors appear to show similar ligand-independent activation, which differs from the LH receptor, which is reported only to show ligand-independent activity under nonphysiological conditions (32).

Two generations of Leydig cells arise during normal testis development: a fetal population, which appears shortly after testis differentiation *in utero*, and an adult population, which arises shortly before puberty (starting around d 5–10 in the mouse) (33, 34). In both FSHβKO and FSHRKO mice, Leydig cell number and testicular testosterone levels were normal on d 1 and 5, showing that FSH and the FSHR do not appear to play a role in the development of the fetal population. During fetal growth *in utero*, Leydig cell function develops normally in GnRH-deficient mice (1) and in mice with a null mutation in the common α-subunit (35), and it is not unexpected, therefore, that Leydig cell function is normal on the day of birth in FSHβKO and FSHRKO mice. Shortly after birth, fetal Leydig cells become critically dependent on gonadotropins (1), but results reported here show that neither FSH nor its receptor is required at this time.

Within the testis only the Sertoli cell population expresses the FSHR, and the effect of the FSHR null mutation on Leydig cell development must, therefore, be mediated through Ser-

toli cells. This implies that factors released by Sertoli cells act to induce normal Leydig cell proliferation/differentiation. From other studies of mutant mice two Sertoli cell-derived factors (desert hedgehog and platelet-derived growth factor A) have been implicated in this process (36, 37). It is likely that the FSHR is a permissive factor in this process, ensuring that overall Sertoli cell activity is high enough to maintain normal output of the trophic factors. It is clear, however, that FSH is required for normal levels of Sertoli cell activity, because in both FSHβKO and FSHRKO mice cell number is reduced, and spermatogenesis is compromised (28, 38).

Given that LH levels, Leydig cell number, and testosterone levels are normal in FSHβKO mice, it is not clear why intratesticular testosterone levels fail to increase normally after hCG stimulation. It may simply be due to a physical effect of the 50% reduction in size of the FSHβKO testis, which will affect blood flow to the testis interstitium and may limit the amount of testosterone that can actually be held within the testis. Alternatively, it is possible that the sensitivity of the testis to hCG stimulation or the maximum steroidogenic capacity of the Leydig cells is reduced in the absence of FSH. This does not, however, appear to affect androgen production under normal conditions.

Previous studies showing an effect of FSH injections on Leydig cell activity were carried out using animals with low or absent circulating LH (4–11). Results from this study suggest that in the presence of LH, FSH is not required for normal Leydig cell development as long as FSHR are present. The lack of effect of the FSHβ null mutation on Leydig cell number contrasts with the almost complete failure of postpubertal development of Leydig cell number in GnRH-deficient mice, which lack LH and FSH (39). Thus, LH alone is sufficient for normal postnatal Leydig cell development (in the presence of the FSHR), but in the absence of LH, evidence from earlier studies suggests that FSH can induce stimulation of Leydig cell activity (9, 11).

Acknowledgments

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