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Testosterone Replacement Therapy Induces Spermatogenesis and Partially Restores Fertility in Luteinizing Hormone Receptor Knockout Mice

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Testosterone (T) is essential for spermatogenesis, fertility, and maintenance of the male phenotype. We analyzed in hypogonadal LH receptor knockout (LuRKO) male mice whether T treatment can restore their phenotype, spermatogenesis, and fertility. In LuRKO mice, spermatogenesis is arrested at round spermatids, adult-type Leydig cells are absent, T production is dramatically decreased, the animals are cryptorchid, and their accessory sex organs are atrophic. T replacement therapy from 21 d of life for 60 or 120 d in LuRKO mice induced a male phenotype macroscopically indistinguishable from that of wild-type littermates as well as full spermatogenesis and testicular descent. Thus, the absence of LH-dependent prepubertal androgen priming is not necessary for subsequent maturation of the male phenotype. Conspicuously, some abnormalities remained in epididymal histology after T treatment despite normal expression of several epididymis-specific genes in caput epididymis. The mice displayed normal mating behavior, although at lower frequency than wild-type controls. The spermatozoa were able to fertilize oocytes, but their impaired passage from epididymis to uterus was apparent. The mice remained subfertile, because only 9% of all breedings resulted in pregnancy, and only two of 13 mice (15%) were fertile. Moreover, inflammation in epididymides and prostate was found in many T-treated LuRKO mice, which probably impaired sperm transport and contributed to their high rate of subfertility. In conclusion, T replacement initiated prepubertally only partially restores the fertility of LuRKO mice, even though most features of the male phenotype recover. Full fertility may require higher and/or earlier postnatal T exposure or production of other Leydig cell factors lacking in this model. (*Endocrinology* 146: 596–606, 2005)

THE PIVOTAL ROLE of androgens in male fertility is well established (1, 2). The main testicular androgen, testosterone (T), is produced by Leydig cells under the stimulation of pituitary LH. LH secretion, in turn, is regulated by hypothalamic GnRH, the most proximal effector of the hypothalamic-pituitary-gonadal axis. LH together with testicular auto- and paracrine factors is responsible for the regulation of balanced sex hormone production and gametogenesis (3, 4).

Several transgenic and knockout mouse models have recently been developed to clarify details of function of the hypothalamic-pituitary-gonadal axis in normal gonadal function and its disturbances (5–9). One such model is the LH receptor (LHR) knockout (LuRKO) mouse (10–12). In the model developed by us (10), targeted deletion of exon 11 of the LHR totally inactivates LH/LHR function, preventing postnatal, but not prenatal, Leydig cell maturation and androgen production, consequently leading to hypergonadotropic hypogonadism. The phenotype of LuRKO males includes infertility due to cryptorchidism and arrest of spermatogenesis at the round spermatid stage. In addition, the abolished androgen production causes atrophy of accessory sex organs and lack of normal sexual behavior. The LuRKO mouse model thus allows us to study the consequences of deficient postnatal Leydig cell function caused by LHR inactivation and the importance of factors originating from these cells to male reproductive functions *in vivo*.

Other genetically modified mouse models have revealed valuable information about the effects of missing FSH (8, 9) and FSH receptor (7) function and the lack of both gonadotropins (5, 6). FSH β-subunit knockout mice have demonstrated that FSH action is essential for normal Sertoli and germ cell number, with a direct influence on testis size. Furthermore, the lack of FSH reduces sperm motility, although the mice were reported to retain full fertility (8, 9). In addition to reduced testis size, spermatogenesis, and sperm motility, FSH receptor knockout mice (7) were reported to produce reduced T levels and display aberrant sperm morphology and reduced fertility. In the case of hypogonadal *hpg* mice (5), in which the expression of both gonadotropins is suppressed because of inactivating deletion of the GnRH gene, the hormone deficiency leads to cryptorchidism, arrest of spermatogenesis, and infertility. These mouse models have shown that both gonadotropins are essential for normal reproductive function in males, but only LH action is absolutely necessary for the maintenance of fertility (10).

In the present study we subjected LuRKO mice to high dose T replacement, starting at prepubertal age and assessed

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Abbreviations: BW, Body weight; LHR, LH receptor; LuRKO, LH receptor knockout; T, testosterone; WT, wild-type.

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its effects on sexual maturation, including spermatogenesis and fertility, in adulthood. We also monitored the effects of T on growth and maturation of the accessory sex organs. Previous studies in gonadotropin-deficient *hpg* mice have shown that spermatogenesis can be induced by exogenously administered androgens (6, 13), but due to specific inactivation on LH function without effects on FSH action, LuRKO mice offer a more appropriate model to study the consequences of complete and specific loss of LH action.

Materials and Methods

Animals

Basic characteristics of the reproductive phenotype of LuRKO mice have been described previously (10, 12, 14). The animals were housed under controlled environmental conditions (12 h of light/12 h of darkness; temperature, 21 ± 1 C) in the animal facility of University of Turku. They were fed mouse chow SDS RM-3 (Special Diet Service, E, soy-free, Whitman, Essex, UK) and tap water *ad libitum*. All procedures were carried out according to the institutional policy of University of Turku. Mice were genotyped using the PCR method described previously (10).

T replacement

For T replacement therapy, we prepared 1-cm capsules filled with T powder (Fluka Chemie AG, Buchs, Switzerland). We used SILASTIC brand tubing (Dow Corning, Inc., Midland, MI; inner diameter, 1.98 mm; outer diameter, 3.18 mm) sealed at both ends with SILASTIC adhesive (Elastosil RTV-1 Silicone Rubber, Wacker-Chemie GmbH, Munich, Germany). Capsules were implanted subdermally under anesthesia into homozygous LuRKO mice at the age of 21 d. Wild-type (WT) littermates, implanted with empty capsules, were used as controls. Anesthesia was performed using 100–300 μl 2% tribromoethanol (Avertin) (15), and buprenorphine (Temgesic; 3–5 μg/mouse sc.) was used for postoperative analgesia. The animals were weighed, and their ano-genital distances were measured weekly. After 8 wk of implantation, the animals were killed by cardiac exsanguination under anesthesia. Blood was centrifuged, and sera were stored at −20 C until analyzed. One testis per mouse was snap-frozen in liquid nitrogen and stored at −70 C. The other testis and epididymides, vasa deferentia, and prostate blocks were fixed for histological analyses. Several other organs (liver, spleen, adrenals, kidneys, and seminal vesicles) were weighed.

Hormone assays

The serum samples were analyzed for LH and FSH using immunofluorometric assays as described previously (16, 17). For serum T determination, 25-μl aliquots were extracted twice with 2 ml diethyl ether and evaporated under nitrogen to dryness. The residues were reconstituted in PBS and measured using standard RIA, as described previously (18). Testicular T levels were measured by homogenizing a weighed aliquot of the testis tissue (approximately one half of LuRKO testis and one quarter of WT testis) in 500 μl PBS. A 100-μl aliquot of the homogenate was subjected to extraction with diethyl ether, and T RIA was performed (18). Protein concentrations of the testicular homogenates were determined using the Bradford method (19), and testicular T levels were expressed per weight unit of protein.

Histological analyses

The testes, epididymides, vasa deferentia, and prostate blocks were fixed in 4% paraformaldehyde at 20 C for 10–20 h, then dehydrated, embedded in paraffin, and sectioned at 5-μm thickness. Sections were stained with hematoxylin and eosin (Delafield's).

Measurement of tubule diameters, interstitial cell volume density, and spermatozoa

Tubule diameters and interstitial cell areas (volume density) of the testis samples were measured from paraffin sections using the Leica IM1000 program (Leica, Heerbrugg, Switzerland). Sections from three or four animals per group were analyzed, and all tubules in one section, cut from the middle of the testis, were measured. Similarly, interstitial cell areas of the sections were measured and compared with total section area, and relative interstitial cell mass was calculated.

The number and motility of spermatozoa in cauda epididymis were analyzed from six T-treated and six WT mice by immersing the tissue into KSOM-AA medium, designed specially for mouse embryo culture (catalogue no. MR-121, Specialty Media, Phillipsburg, NJ), followed by incubation at 37 C for 15 min. The motility was compared with that of spermatozoa of WT littermates. The number of spermatozoa was counted under a microscope using the Bürker chamber.

RNA analysis

Total RNA from snap-frozen caput epididymides was isolated using the single-step method (20). The expressions of epididymis-specific genes, glutathione peroxidase 5 (GPX 5), mouse epididymal protein 17 (MEP-17), murine epididymal retinoic acid-binding protein (mE-RABP), serine protease inhibitor, Kunitz type 4 (Spint4), and ribonuclease A family 9 (RNase9) (21, 22), were analyzed using RT-PCR. RT and PCRs were performed in the same tube, and 1 μg deoxyribonuclease I (amplification grade, Invitrogen Life Technologies, Paisley, UK)-treated RNA was used in each PCR. Avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI) was used for the reverse transcriptase reaction (50 C, 10 min), and Dynazyme II-polymerase (Finnzymes, Espoo, Finland) was used for the amplification reaction. Primers and annealing temperatures are presented in Table 1.

Fertilization assays

For the fertilization analysis, LuRKO mice were treated with T until postnatal d 120, and 13 T-treated LuRKO and six WT male mice were used for analysis. A total of 78 young WT FVB/N female mice were stimulated by the standard superovulation method (10 IU pregnant mare's serum gonadotropin, ip injection, followed by a 5-IU human chorionic gonadotropin injection 47 h later) and bred with T-treated LuRKO males. Six females were similarly stimulated and bred with WT males. Vaginal plugs, indicating successful mating, were observed the following morning, and the number of oocytes recovered from oviducts was calculated from a total of 41 females bred with T-treated LuRKO males and from six females bred with WT males. The remaining animals, 37 females bred with T-treated LuRKO males, were followed up to 3 wk to analyze their pregnancy rate. All T-treated LuRKO males examined were bred two to eight times with different females, caging one female with one male at a time. For oocyte calculation, female mice were killed in the morning after mating, and oviducts were placed into FHM medium (Specialty Media). After dispersing the oocyte-cumulus cell mass by hyaluronidase treatment, the number of oocytes was calculated under a stereomicroscope. Fertilized oocytes were detected as having two pronuclei and were cultured until the morula stage. To identify ejaculated spermatozoa of LuRKO males after matings, vaginal plugs and lumens of uteri were soaked in FHM medium and analyzed under a microscope to detect spermatozoa.

Statistical analyses

The SigmaStat program (SPSS, Inc., Chicago, IL) was used for statistical analyses. ANOVA and *t* tests were used. The limit of significance was set at $P < 0.05$, and the data are presented as the mean ± SE for hormone levels and as the mean ± SD for weights.

Results

External genitals

During T treatment, the underdeveloped external genitals (penis and scrotum) of the LuRKO mice grew to the size of those in WT mice, and the testes descended into the scrotum. Furthermore, the ano-genital distances increased and were not significantly different from those in WT mice (Fig. 1 and Table 2). After T treatment, LuRKO mice could not be distinguished externally from WT mice.

TABLE 1. Primers used in RT-PCR for the analysis of epididymal gene expression

Gene	Primer sequence	Temperature (C)	GenBank accession no.
Mouse epididymal protein, MEP 17	5′-TGACCAAAAACTGGTGCTGA-3′ 5′-GGCTCATCCTCGTTCTCAAG-3′	55	AF082221
Glutathione peroxidase 5, GPX5	5′-CTTCTAGCCAGCTATGTG-3′ 5′-GTACTGGATTGTCAGACCG-3′	55	M68896
Murine epididymal retinoic acid-binding protein, mE-RABP	5′-GTTTTTAGGCTTCTGGTATGA-3′ 5′-CTGATATTCTGGTGACCTTGTA-3′	56	U68381
Serine protease inhibitor, Kunitz type 4, Spint4	5′-GCAATGGCAACCTTAATAAC-3′ 5′-TCAGGTTCAGATTCAGGTTC-3′	55	AY226988
RNase9	5′-GCAAGAGTCTGGTGAAGAGT-3′ 5′-AGTCCTGAGTTCAGTGTTGC-3′	55	AY226989
β-Actin	5′-CGTGGGCCGCCCTAGGCACCA-3′ 5′-TTGGCCTTAGGGTTCAGGGGG-3′	54	NM_007393

Body and organ weights

Body weights (BW) of the mice were measured weekly after implantation of T capsules. At the time of implantation (3 wk of age), there were no significant differences between the weights of LuRKO and WT mice, but the mice in the T-treated LuRKO group were somewhat heavier than the others at the beginning of the treatment. After T treatment, at the age of 11 wk, there was no statistically significant difference between T-treated and untreated LuRKO mice (26.3 ± 2.1 *vs.* 27.2 ± 4.5 g, respectively), but the WT mice were significantly heavier (32.1 ± 4.3 g; $P < 0.05$) than the other two groups (Fig. 1 and Table 2).

The relative testis weights of the untreated LuRKO mice were significantly lower compared with WT mice (0.8 ± 0.13 *vs.* 3.2 ± 0.41 mg/g BW), but after T treatment they were indistinguishable from those of WT mice (3.0 ± 0.45 mg/g BW). Also, the hypoplastic accessory sex glands of LuRKO mice (epididymides, seminal vesicles, and prostate) grew markedly during T treatment, reaching the size of those in WT mice (epididymides, 1.2 ± 0.11 *vs.* 1.1 ± 0.07 mg/g BW, respectively). The seminal vesicles exceeded (Fig. 2 and Table 2) and prostates (data not shown) reached the weights of the respective WT organs.

With respect to other tissue weights (Table 2), as previously shown in *hpg* mice (6), the kidney weights of LuRKO mice displayed a marked response to the elevated T levels, increasing above the WT weights (T-treated LuRKO *vs.* LuRKO *vs.* WT, 9.7 ± 0.80 *vs.* 4.2 ± 0.47 *vs.* 5.7 ± 0.65 mg/g BW). The liver and pituitary weights did not change during T treatment; spleen size was increased in LuRKO mice, but T treatment normalized it. The adrenal weights were largest in untreated LuRKO mice (0.11 ± 0.02 mg/g BW), and after T treatment, their sizes were reduced, but remained significantly larger (0.09 ± 0.02 mg/g BW) than those in WT mice (0.06 ± 0.02 mg/g BW). Furthermore, it was evident that the bulbourethral glands, macroscopically undetectable in untreated LuRKO mice, appeared after T treatment (Fig. 2).

Hormone levels

The results of hormone measurements are shown in Fig. 3. The T capsules were expected to increase serum T levels above the normal range of WT male mice. Accordingly, the mean T level in the treated LuRKO mice was 32.5 ± 3.99 nmol/liter, which was about 8-fold higher than that in WT mice (4.1 ± 1.10 nmol/liter). In untreated LuRKO males, the T levels were profoundly lower, although still detectable (0.6 ± 0.22 nmol/liter). The mean testicular T levels of T-treated LuRKO mice were elevated compared with those of untreated LuRKO mice (0.6 ± 0.22 *vs.* 0.2 ± 0.07 nmol/g protein), although the difference did not reach statistical significance. The levels in WT mice were notably higher (5.5 ± 0.69 nmol/g protein).

The serum LH levels in LuRKO mice were markedly higher than those in WT animals (4.2 ± 0.92 *vs.* 0.1 ± 0.04 μg/liter). In LuRKO mice, LH decreased dramatically after T treatment to nearly undetectable levels (0.01 ± 0.003 μg/liter), demonstrating a normal feedback response to T at the hypothalamic-pituitary level. Serum FSH levels in LuRKO mice were elevated compared with those in WT mice (68.0 ± 6.87 *vs.* 22.8 ± 2.07 μg/liter), and T treatment suppressed these levels (10.2 ± 1.56 μg/liter) to about 50% of WT male concentrations.

Testicular and accessory sex organ histology, morphometry, and expression of epididymis-specific mRNAs

Histology of the LuRKO testes (Fig. 4) showed that the seminiferous tubules were narrow, and spermatogenesis was arrested at the round spermatid stage, as shown previously (10). The number and size of Leydig cells was decreased in the interstitial space compared with the levels of WT mice, and this was confirmed quantitatively by counting the interstitial cell mass per testis (0.13 ± 0.02 *vs.* 1.21 ± 0.35 mg/testis). After T treatment the width of seminiferous tubules increased, and full spermatogenesis could be observed in the testes, but the mass of interstitial cells, reflecting the amount of Leydig cells, remained unaltered (0.17 ± 0.04 mg/testis; Fig. 4 and Table 3). The diameter of seminiferous tubules was 93.5 ± 11.1 μm in the LuRKO testes, and it increased during T treatment to nearly the size of WT tubules (148.7 ± 16.8 *vs.* 159.1 ± 19.4 μm, respectively). However, a statistically significant difference remained between T-treated LuRKO and WT males (Table 3).

The histology of the epididymides (Fig. 5) showed that ep-

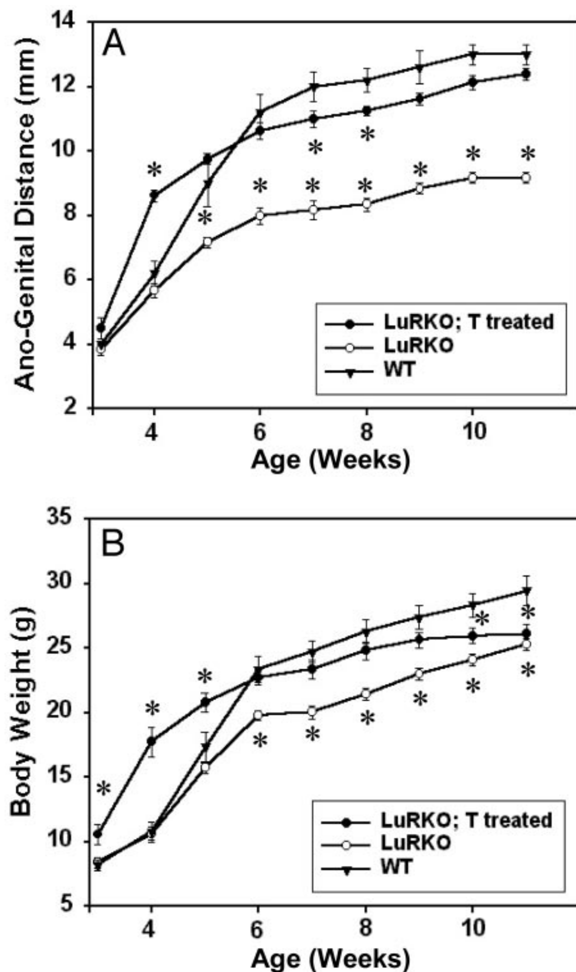

FIG. 1. The ano-genital distances (A) and body weights (B) of LuRKO mice, T-treated LuRKO mice, and WT controls. The *asterisks* indicate statistically significant differences from the age-matched WT group ($P < 0.05$). There were five to eight mice per group.

ithelial cells in the caput region of T-treated LuRKO mice were partially disorganized, and all principal cells did not follow the normal arrangement around the lumen. Also, many vacuoles existed in the epithelium of caput epididymis. In more distal parts of the epididymis, corpus and cauda histology appeared normal. However, there was considerable variation between different mice in the histology and amount of spermatozoa.

Analysis of epididymis-specific genes (*GPX5*, *MEP-17*, *mE-RABP*, *Spint4*, and *RNase9*) by RT-PCR showed recovery in the expression of the genes by T replacement therapy, also indicating that many aspects of the epididymal function were restored (Fig. 5). Because of undeveloped epididymides, tissue was not obtained for these measurements from untreated LuRKO mice.

Histology of the prostate block in untreated LuRKO mice showed the presence of only small glands of poorly developed prostates. Small seminal vesicles and coagulating glands could also be detected. The histology of the prostate resembled the undifferentiated state of newborn pups. After T treatment, prostates of the LuRKO mice grew considerably and appeared histologically normal. Also the seminal vesicles, coagulating glands, and vasa deferentia attained normal histological appearance, and the glandular lumina and vasa deferentia appeared open (Fig. 6). However, contrary to the histology of WT mice, four T-treated LuRKO mice of six presented with vigorous inflammation in prostate, coagulating glands, and vasa deferentia, as indicated by the abundant presence of lymphocyte and neutrophilic cells. Some acini in prostates of T-treated LuRKO mice were filled with necrotic mass, and the epithelial architecture of these acini was disturbed, whereas some acini indicated normal histological appearance. Abundant lymphocyte accumulation was also detected in stromal tissue and between tissue layers in prostates and vasa deferentia, and the inflammation was associated with abnormal epithelial structure of vasa deferentia (Fig. 6). Furthermore, some mice developed severe inflammation in the epididymis, indicated by the accumulation of necrotic cell masses (data not shown).

Motility and number of spermatozoa

The number of spermatozoa in cauda epididymis was lower and highly variable in T-treated mice compared with WT mice (3.2 ± 3.2 *vs.* $12.4 \pm 3.2 \times 10^6$ spermatozoa/cauda), indicating that quantitative spermatogenesis was incompletely restored by T supplementation (Table 3). The highest level of spermatozoa in T-treated LuRKO mice reached the lowest level of spermatozoa found among WT mice, whereas few spermatozoa were found in cauda epididymis of one mouse of this group. The motility of spermatozoa was analyzed subjectively under a microscope, and no apparent difference between the two groups could be detected. The shape of the spermatozoa also appeared normal in the T-treated mice.

Fertilization assays

Fertility analysis showed, as expected, that all six WT males bred with WT females mated, and vaginal plugs and fertilized oocytes were found in all females. In contrast, only 46% (six of 13) of T-treated LuRKO males were able to ejaculate, as determined by vaginal plugs. Among those mice able to ejaculate, the ejaculating frequency was also decreased. These dysfunctions resulted to a fact that only 23% (18 of 78) of females, bred with T-treated LuRKO males, had vaginal plugs (Table 4). The reduced ejaculation capability

TABLE 2. Body and organ weights of 11-wk-old LuRKO, T-treated LuRKO, and WT mice after an 8-wk treatment with T

	LuRKO	LuRKO T-treated	WT
BW (g)	27.2 ± 4.5^a	26.3 ± 2.1^a	32.1 ± 4.3^b
Testis (mg/g BW)	0.8 ± 0.1^a	3.0 ± 0.5^b	3.2 ± 0.4^b
Ano-genital distance (mm)	9.2 ± 0.4^a	12.4 ± 0.5^b	13.0 ± 0.7^b
Epididymis (mg/g BW)	$—^a$	1.2 ± 0.1^b	1.1 ± 0.1^b
Seminal vesicle (mg/g BW)	$—^a$	10.6 ± 4.1^b	5.7 ± 1.0^c
Pituitary (mg/g BW)	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Adrenal (mg/g BW)	0.11 ± 0.02^a	0.09 ± 0.02^a	0.06 ± 0.02^b
Liver (mg/g BW)	39.4 ± 5.7	42.9 ± 6.9	38.4 ± 3.2
Spleen (mg/g BW)	3.4 ± 0.2^a	2.4 ± 0.6^b	2.6 ± 0.5^b
Kidney (mg/g BW)	4.2 ± 0.5^a	9.7 ± 0.8^b	5.7 ± 0.6^c

Groups with *different superscripts* (^{a} and ^{b}) are significantly different ($P < 0.05$; n = 6–20). Values given as the mean ± SD. —, Too small to be detected.

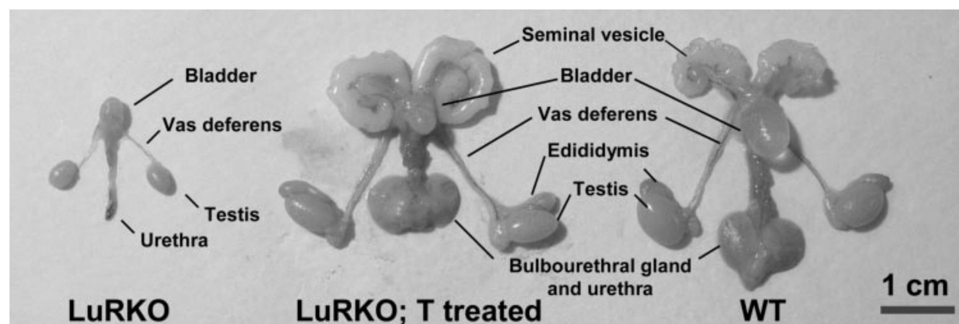

FIG. 2. The urogenital blocks of LuRKO, T-treated LuRKO, and WT mice.

was also confirmed during the breeding tests, in which mating behavior was confirmed for 15 breedings by visual observation. However, the following morning only 47% (seven of 15) of these females were observed to have vaginal plugs. All of these data point to the fact that despite apparently normal sexual behavior, LuRKO males were not able to ejaculate frequently. Furthermore, only in 9% (seven of 78) of the females bred with LuRKO males were found to be fertilized, having fertilized oocytes (three of 41) or delivering offspring (four of 37). The four offspring produced were by two of the 13 T-treated LuRKO males tested, indicating that T replacement therapy recovered fertility only in 15% (two of 13 males) of the treated LuRKO mice. This was in line with the fact that spermatozoa were found in vaginal plugs only occasionally, and when found, few spermatozoa were detected. However, the average litter size and the number of fertilized oocytes detected in the oviducts of females mated with T-treated LuRKO males varied within the normal range, and the offspring were healthy and grew normally.

Discussion

Previous reports of knockout mouse models have confirmed that the actions of both gonadotropins, LH (10–12) and FSH (7), are important for normal reproductive development and function. Disruption of the LHR gene by targeted deletion of exon 11 (LuRKO mouse) brings about infertility and several reproductive organ abnormalities in male and female mice (10). A similar phenotype has been achieved in another model with targeted deletion of exon 1

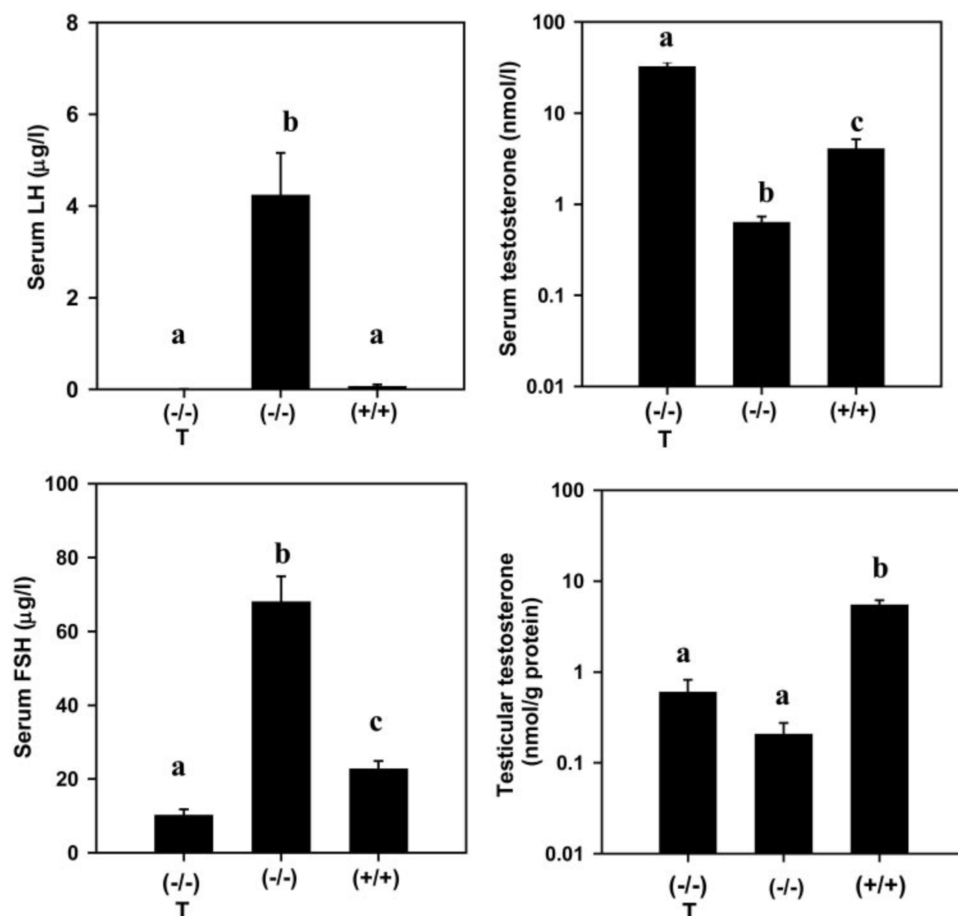

FIG. 3. Serum LH, FSH, and T and testicular T concentrations in T-treated LuRKO (−/− T), LuRKO (−/−), and WT (+/+) mice (n = 5–9 mice/group). The groups marked by *different letters* (a and b) are significantly different ($P < 0.05$).

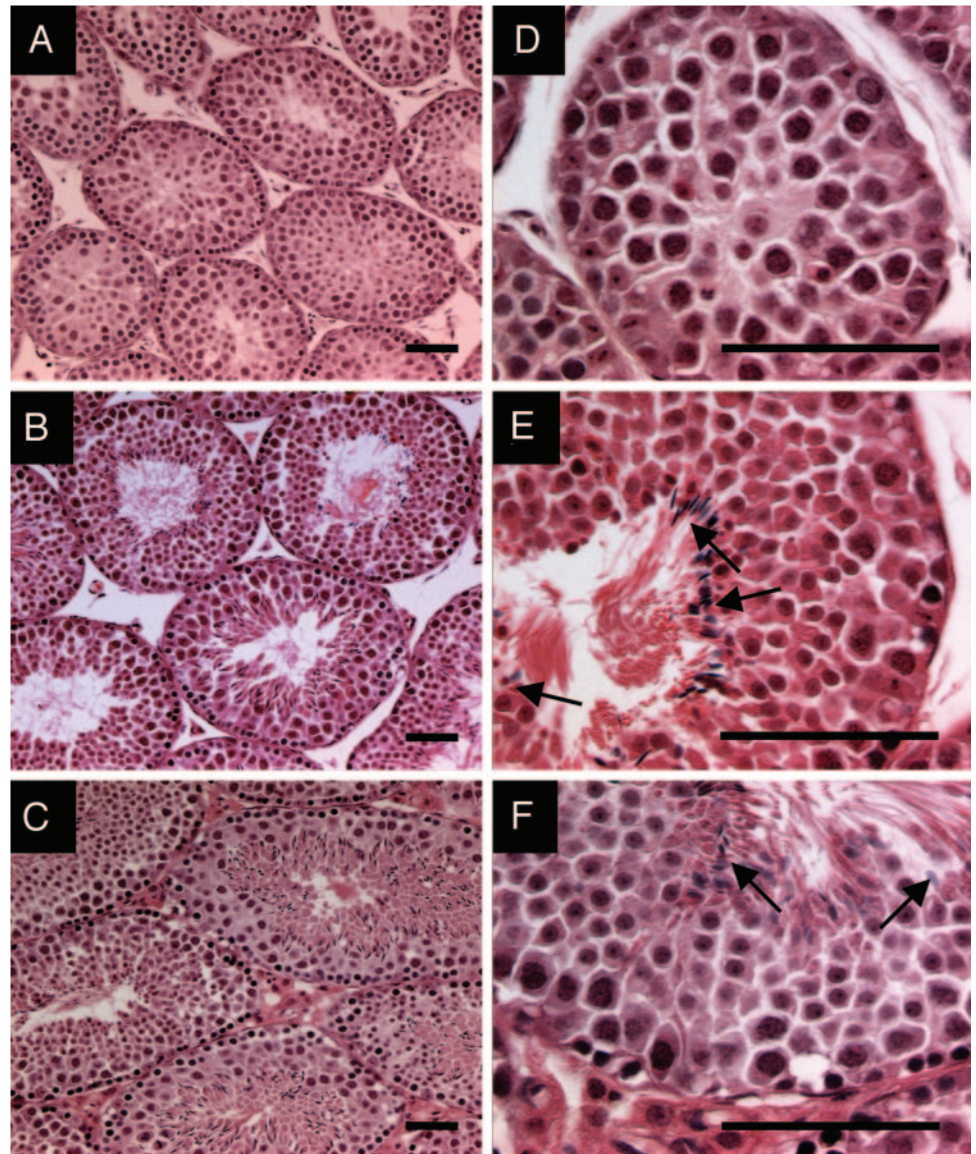

FIG. 4. Representative light microscopic images of testis sections from LuRKO (A and D), T-treated LuRKO (B and E), and WT (C and F) mice. D–F are at higher magnification. *Arrows* indicate elongated spermatids in tubules of T-treated and WT mice. The absence of Leydig cells between tubules is evident in both LuRKO and T-treated LuRKO mice. *Bar*, 50 μm.

of LHR (11). The LuRKO model allows us to selectively address the specific role of LH in the development and function of reproductive organs and fertility, without simultaneous suppression of FSH action, which occurs when gonadotropin-deficient (*hpg*), (6) or GnRH antagonist-treated animals (23) are studied.

In the present study we analyzed whether the anatomical and functional abnormalities of LuRKO mice could be restored by T supplementation. We administered T using a type of subdermal SILASTIC implant, which provided a circulating T concentration that was about 8-fold higher than that in WT control males. This size of the SILASTIC implants has previously been shown to result in the highest spermatid counts, maximum testis weights, and largest tubular diameters in *hpg* mice (6), indicating that the implant size is optimal for T substitution in hypogonadal male mice. The treatment was started at the prepubertal age of 21 d, with the purpose of mimicking the normal onset of puberty. The capsules were kept in place for 60 or 120 d, which exceeds the length of the 35-d spermatogenic cycle of the mouse (24).

TABLE 3. Effect of 8-wk T treatment of LuRKO mice on tubular diameters, interstitial cell areas, and spermatid counts

	Tubular diameter (μm)	Interstitial cells (interstitial cell area/total area; %)	Interstitial cells (interstitial cell area/total area × testis weight; mg/testis)	Spermatid count (10^6/cauda epididymidis)
LuRKO	93.5 ± 11.1^a	0.58 ± 0.12^a	0.13 ± 0.02^a	0^a
LuRKO, T-treated	148.7 ± 16.8^b	0.23 ± 0.06^a	0.17 ± 0.04^a	3.17 ± 3.16^b
WT	159.1 ± 19.4^c	1.11 ± 0.32^b	1.21 ± 0.35^b	12.38 ± 3.23^c

n = 4 Mice (tubular diameters), n = 3 (interstitial cell area), and n = 6 (spermatid counts); in total 654–819 diameters of tubules were measured/group. The values shown are the mean ± SD. The groups with *different superscripts* are significantly different ($P < 0.05$).

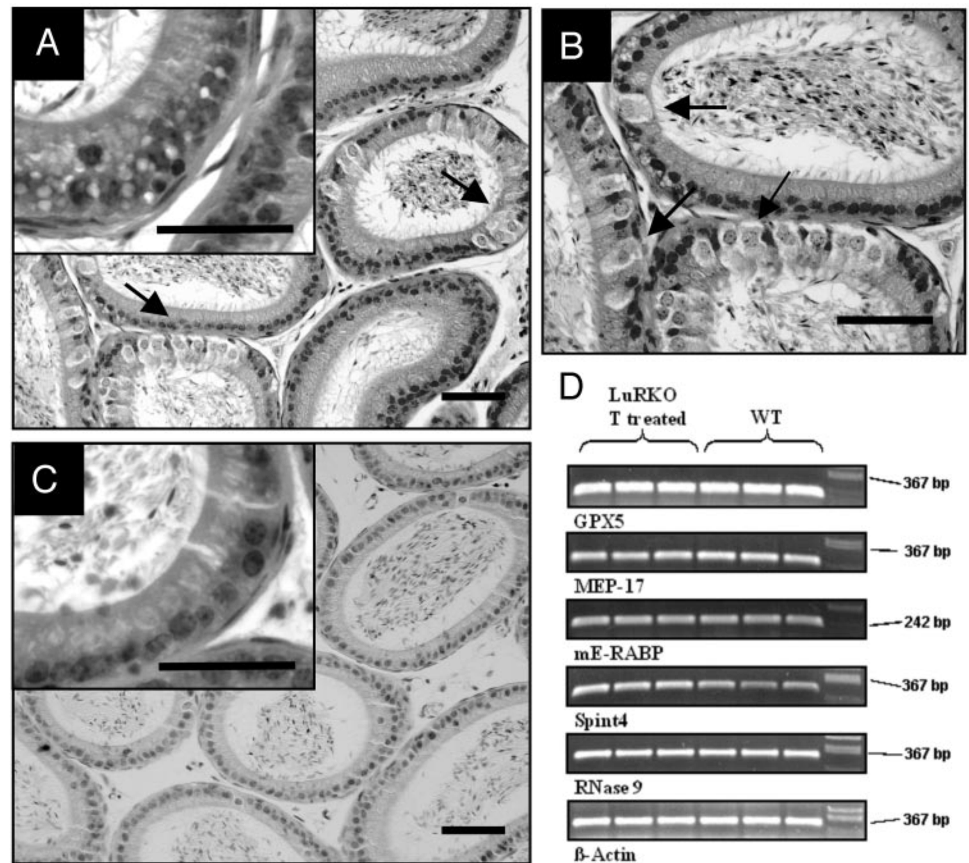

FIG. 5. Representative light microscopic images of caput epididymis sections from T-treated LuRKO (A and B) and WT (C) mice. Histology indicates that the recovery of epididymal morphology is incomplete, as indicated by disordered localization of nuclei in the principal cells of caput epididymis and multiple vacuoles (indicated by *arrows*) in the epithelium. *Bar*, 50 and 25 μm in the *insets* of panels A and C. D, RT-PCR analysis of epididymis-specific genes expressed in cauda epididymis. β-Actin was used as a control.

LuRKO mice showed a clear response to T treatment. Their poorly developed external genitals grew, and the mice could not be distinguished macroscopically from WT controls at the end of treatment. The testes of LuRKO mice, which normally are small (~18% of WT testes) and located in the lower part of the abdominal cavity, descended and reached a normal size. In contrast to T-treated *hpg* mice (13, 25), although the testes descended into the scrotum in both models, only those in T-treated LuRKO mice attained a normal size. This can be explained by the normal FSH action in LuRKO mice, which is necessary for postnatal Sertoli cell proliferation, a prerequisite for attaining normal testis size in adulthood. The levels of both gonadotropins remained dramatically reduced in *hpg* mice, whereas in T-treated LuRKO mice FSH remained at 50% of WT levels.

Testicular descent confirmed the essential role of T in the passage of the testes from the abdominal cavity to the scrotum (26) and indicated that the other Leydig cell product, Insl3, is not essential for transinguinal testis descent. This conclusion arises from the finding that the testes of LuRKO mice express Insl3 at a very low level after 20 d of age, because of their lack of the adult-type Leydig cell population (12). On the contrary, Insl3 is essential for the transabdominal testicular descent from the upper to the lower part of the abdominal cavity (27, 28). The first transabdominal part of testicular descent, dependent on Insl3, occurs in the mouse in fetal life (26), and it is also normal in LuRKO mice (12). The prenatal sexual development of LuRKO mice is normal, and testicular endocrine function before birth appears to be independent of gonadotropin regulation (10), as also shown in *T/ebp/Nkx2.1* knockout mice, which totally lack pituitary development (29), and in glycoprotein hormone common α-subunit knockout mice deficient in LH, FSH, and TSH (30). Apparently the fetal type of Leydig cell, although responsive to LH, is under sufficient stimulation by testicular paracrine factors during the prenatal period to produce proper amounts of T and Insl3 to achieve normal masculinization of male LuRKO fetuses (31–33).

The poorly developed accessory sex organs of LuRKO mice developed during T treatment. Epididymal histology indicated that T treatment largely, although not completely, restored the structural features of this organ, as was also recently reported with another LuRKO model (34). Accordingly, mRNAs of several androgen-dependent and epididymis-specific genes were expressed in this organ after T treatment. With regard to nonreproductive organs, the slightly increased size of the spleen in LuRKO mice was normalized by T treatment, but the kidney size increased above that in WT mice, in keeping with the widely known growth stimulatory effect of T on mouse kidneys (35, 36). Also, the adrenals showed slight changes in weight, being largest in untreated LuRKO mice and decreasing after T treatment. The same effect has previously been noticed in orchidectomized rats, whose adrenal cortex is enlarged after orchidectomy, with an opposite effect of T (37).

T replacement therapy induced qualitatively full spermatogenesis in LuRKO mice, which in untreated LuRKO mice is arrested at stage 6 round spermatids (10). T is known to be

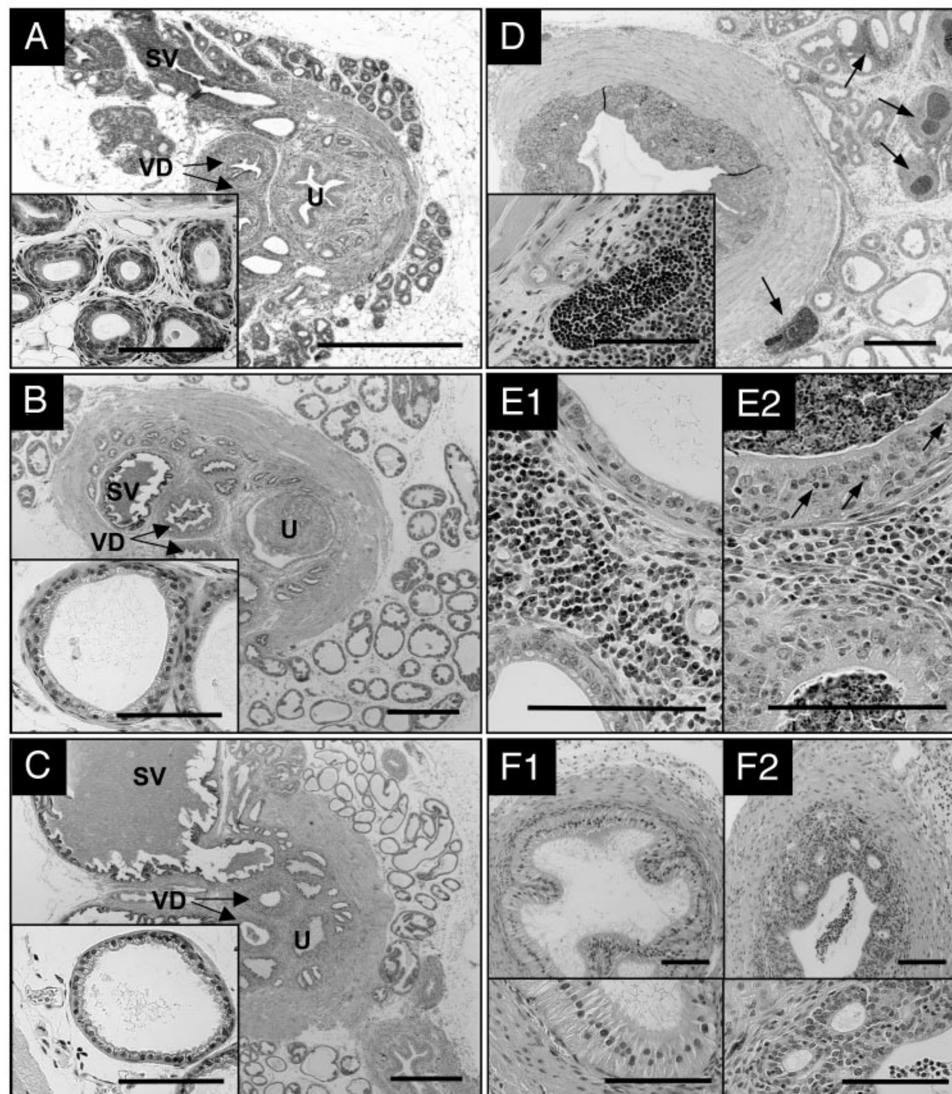

FIG. 6. Representative light microscopic images of prostatic histology of LuRKO (A), T-treated LuRKO (B), and WT (C) mice. The structures of acini are shown in the *insets*. D, Inflammation of prostate in a T-treated LuRKO mouse; leukocyte accumulation is shown in the *inset*. Acini with normal single-layer columnar epithelium, surrounded by stromal leukocyte infiltration (E1), as well as acini with abnormal epithelial architecture and accumulation of luminal leukocytes and necrotic mass (E2) are present in the same sample. F1, Morphologically normal vas deferens; F2, inflamed and morphologically abnormal vas deferens from the same animal. Urethra (U), seminal vesicles (SV), and opening of vas deferens (VD) are indicated. *Arrows* in D indicate the inflammatory changes, which are presented in *insets* and in E1 and E2. *Arrows* in E2 indicate polymorphonuclear leukocytes in the epithelium of the acinus. *Bar*, 500 μm in A–D and 100 μm in *insets* and in E and F.

essential for spermatogenesis from round spermatids, stage 7 onward (38, 39), and these data are confirmed by LuRKO mice. In contrast, the hormone treatment had no effect on the dramatically decreased number of Leydig cells, whose proliferation is known to be LH dependent. Despite the highly elevated T levels in the circulation, T levels in testes increased only slightly, but still sufficiently to induce full spermatogenesis in LuRKO mice. The normal testis weight, recovered tubular diameter, and appearance of tubular cross-sections indicated the recovery of normal spermatogenesis. However, sperm counts in cauda epididymis remained reduced compared with those in WT mice. Although the number of epididymal spermatozoa was not quantitatively recovered, their shape and motility appeared normal after T treatment. This may be explained by the fact that some factors essential for sperm maturation in epididymis are missing in tubular fluid (40). Previous studies have shown that *in utero* exposure to dioxin causes a similar response, with normal testis size and unaltered daily sperm production, but decreased epididymal sperm count (41). Thus, exposure to dioxin during

TABLE 4. Test breeding and fertility data of T-treated LuRKO mice and WT controls

	Males ejaculated/ tested males		Females with vaginal plugs/tested		Fertile males/ tested males		Females with fertilized oocytes or offspring /All females		Females with fertilized oocytes or offspring /Females with vaginal plugs	
	No.	%	No.	%	No.	%	No.	%	No.	%
LuRKO, T-treated	6/13	46	18/78	23	2/13	15	7/78	9	7/18	39
WT	6/6	100	6/6	100	6/6	100	6/6	100	6/6	100

Fertility of the T-treated LuRKO males was analyzed by analyzing fertilized oocytes and observing pregnancies of females. The same T-treated LuRKO males were used in both analyses, but females were used only once for the analyses. The results of both analyses were equal and are presented together.

development *in utero* has an inhibitory effect on male fertility by decreasing sperm count in epididymis without an effect on testis, causing the discrepancy between testicular and epididymal sperm counts. Also, a recent study on *act* (activator of cAMP-responsive element modulator in testis) gene knockout mice reported similar findings, with normal spermatogenesis and roughly normal testis weight in the face of remarkably decreased epididymal sperm counts (42).

LuRKO mice totally lack masculine sexual behavior, in keeping with their extremely low androgen levels. T substitution was able to partly restore male-type sexual activity of LuRKO males. The breeding tests indicated that about half (46%) of T-treated LuRKO males were able to ejaculate at least occasionally, although the frequency of ejaculations was even more severely reduced. Some factors from seminal vesicles are known to be essential for vaginal plug formation (43, 44); therefore, the possibility exists that the lack of such factors and other possible defects in the accessory sex organs are the reason for the small number of vaginal plugs found in the T-treated LuRKO mice. However, in the present study six mice of 13 were able to produce vaginal plugs, thus demonstrating grossly normal function of the accessory sex glands. The observation that the T-treated LuRKO mice ejaculated irregularly in breeding tests indicated more diminished ejaculation frequency rather than a defect in plug formation. In addition, few spermatozoa were found in vaginal plugs, indicating reduced transfer of spermatozoa from the epididymis to the ejaculate. Accordingly, fertilized oocytes were found only occasionally, also supporting incomplete function of the sperm transport from cauda epididymis.

To conclude, our results indicated that fertility was recovered by T treatment in only two of 13 mice (15%). However, 46% of the T-treated mice demonstrated normal sexual behavior. Hence, although fertility can be restored in some LuRKO mice by T treatment, the majority of the mice remain severely subfertile for reasons that include poor accessory sex gland function and impaired sexual behavior. The gonadotropin-independent androgen production in LuRKO males is roughly normal until d 10 postpartum. Because T replacement in the current study was initiated at 21 d of age, the reproductive phenotype observed in the mice was apparently due to defective androgen exposure between d 10–20 of postnatal life.

Our findings with LuRKO mice differ somewhat from those with T-treated *hpg* mice (6), which remained totally infertile with no copulatory plugs observed in fertilization tests. This suggests that even though spermatogenesis of the androgen-treated *hpg* mice recovered, and spermatozoa were able to fertilize oocytes *in vitro*, the mice had a failure in their mating behavior. This, in turn, has been considered a consequence of the missing neonatal androgen exposure that is known to be important for sexual behavior (45). In another study of rats, Kolho *et al.* (23) showed that neonatal exposure of male rats to GnRH antagonist induced ejaculatory impotence and infertility, which recovered when the animals grew older. A similar situation seems to prevail in LuRKO mice treated with T, although we did not test whether the condition recovered after time. In addition, Lei *et al.* (34) recently demonstrated that fertility in LHR KO mice could not be restored by T substitution, and their studies also indicated that androgen supplementation did not restore all defects of the epididymis caused by LHR inactivation (11). The role of LH in epididymal sperm maturation is also supported by the study by Zhang *et al.* (46) in monkeys, which suggested that LH action could be important for plasminogen activator-dependent sperm maturation in this organ. In the present study the dose of T used was higher and the treatment time was longer than in the study by Lei *et al.* (34), probably explaining the different results obtained. It has been shown previously that in LuRKO mice, prenatal and early postnatal testicular T production is normal and independent of gonadotropins (10, 14). We hypothesize that the postnatal T levels, despite the gonadotropin-independent fetal Leydig cell activity, may still be lower in LuRKO than in WT males, which could explain the incomplete recovery of fertility of the T-replaced LuRKO mice. It is also possible that other putative factors regulating epididymal function, originating from Leydig cells or through their activity on the tubular compartment, are defective in T-treated LuRKO mice. The restoration of normal spermatogenesis by T alone is in keeping with a recent study of *hpg* mice (25) in which both T and hCG replacement similarly initiated full spermatogenesis, providing evidence against the need for Leydig cell factors other than T. However, epididymal function and fertility were not examined in the latter study.

Severe inflammation was observed in the prostates and epididymides of several T-treated LuRKO mice. It is likely that this pathology is related to the disturbance of sperm transport to ejaculated semen in the T-treated males. The inflammation dramatically changed the epithelial structure of prostates and vasa deferentia. In addition, in several mice, inflammation was present in epididymis, obviously disturbing their normal function. The exact mechanisms by which T induces inflammation and/or infection in the urogenital organs of LuRKO mice remain unknown. It has been well demonstrated that excessive exposure to estrogens, given alone or in combination with androgens, causes chronic prostatitis in rodents (47–49), but this has not been reported to occur in animals treated with T alone. In contrast, whether the lack of other Leydig cell-derived factors, *e.g.* Insl3, defective androgen exposure before the onset of replacement therapy, or the supraphysiologically elevated serum T level during treatment is involved remains to be explored.

A role for Insl3 in proper spermatogenesis has been suggested. Insl3 receptors have been identified in germ cells, and this hormone has been shown to reduce germ cell apoptosis (50). In addition, Insl3 expression is increased during puberty, which suggests that Insl3 might play a role in the regulation of fertility (51), even though Zimmermann *et al.* (52) showed that Insl3 expression is not essential for spermatogenesis. Insl3 knockout mice are a complicated model to address the spermatogenic effects of this hormone because of their cryptorchidism. Hence, the possible role of Insl3 in full fertility still remains elusive.

In conclusion, we have shown in the present study that T treatment of LHR KO mice restores most of the reproductive abnormalities caused by the absence of LH-stim-

ulated testicular function. The treatment established full, but quantitatively diminished, spermatogenesis and recovered most accessory sex organ functions. During hormone treatment, some mice attained normal sexual behavior, were able to ejaculate at low frequency, and became fertile. However, T treatment did not fully repair the reproductive defects in most of the LuRKO males, which remained highly subfertile. The cause of the poorly developed fertility seems to be multifactorial, and its details remain to be analyzed further.

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