MECHANISMS IN ENDOCRINOLOGY

Hormonal regulation of spermatogenesis: mutant mice challenging old paradigms

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Abstract

The two pituitary gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and in particular LH-stimulated high intratesticular testosterone (ITT) concentration, are considered crucial for spermatogenesis. We have revisited these concepts in genetically modified mice, one being the *LH receptor* (*R*)-knockout mouse (LuRKO), the other a transgenic mouse expressing in Sertoli cells a highly constitutively active mutated *Fshr* (Fshr-CAM). It was found that full spermatogenesis was induced by exogenous testosterone treatment in LuRKO mice at doses that restored ITT concentration to a level corresponding to the normal circulating testosterone level in WT mice, ≈5 nmol/L, which is 1.4% of the normal high ITT concentration. When hypogonadal LuRKO and Fshr-CAM mice were crossed, the double-mutant mice with strong FSH signaling, but minimal testosterone production, showed near-normal spermatogenesis, even when their residual androgen action was blocked with the strong antiandrogen flutamide. In conclusion, our findings challenge two dogmas of the hormonal regulation of male fertility: (1) high ITT concentration is not necessary for spermatogenesis and (2) strong FSH stimulation can maintain spermatogenesis without testosterone. These findings have clinical relevance for the development of hormonal male contraception and for the treatment of idiopathic oligozoospermia.

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Introduction

The corner stone of the hormonal regulation of spermatogenesis is its maintenance by the high intratesticular concentration of testosterone (ITT) (1, 2). This is achieved through luteinizing hormone (LH) stimulation of Leydig cell steroidogenesis, after which testosterone activates in paracrine fashion in Sertoli cells the production of an array of other paracrine factors that maintain the differentiation and proliferation of spermatogenic cells (1, 2, 3). ITT is 50- to 100-fold higher than that present in peripheral circulation

Invited Author's profile

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(4, 5, 6), and this high concentration is considered crucial for the maintenance of effective spermatogenesis. The need of such high testosterone concentration is not easy to comprehend, because apparently the same androgen receptor (AR) that is activated by nanomolar concentrations of testosterone in extragonadal tissues (7) should not need orders of magnitude higher testosterone concentration to be activated in the testis. The question about the level of ITT that is needed for spermatogenesis is important, because attempts to reduce it below that critical level are in the focus of the development of a hormonal contraceptive for men (2, 8).

The other unsolved conundrum in the hormonal regulation of spermatogenesis concerns the role of FSH. We described some time ago the phenotype of five men with inactivating mutation of the *FSHR* gene (9). The men were subfertile (two had two children each) with small testes, though with considerable variation in size, but conspicuously, none was azoospermic. This indicated that spermatogenesis is possible without FSH, which finding was against the older dogma that FSH is needed for the pubertal initiation of spermatogenesis (1, 10). The human phenotype in FSHR inactivation was subsequently confirmed by knockout mice (KO) for *Fshb* and *Fshr* (11, 12, 13): besides somewhat smaller testes the males in both animal models had full spermatogenesis and fertility. The only genetic model promoting the necessity of FSH for qualitatively complete spermatogenesis is the phenotype of the men with inactivating *FSHB* mutation; all five men so far reported are azoospermic (reviewed in 14). Hence, it remains unclear whether FSH is really needed for the initiation and/or maintenance of spermatogenesis, besides probably supporting testosterone in the maintenance of qualitatively and quantitatively normal spermatogenesis.

We have previously produced genetically modified mice to address the two questions presented above: (1) what is the minimum ITT concentration to maintain spermatogenesis and (2) what is the role of FSH in spermatogenesis? The mouse models used to produce the data to be reviewed below are the *Lhr*-KO mouse (LuRKO) (15) and a transgenic mouse expressing a constitutively activating mutant of *Fshr* in Sertoli cells (Fshr-CAM) (16).

The necessity of high ITT for spermatogenesis

The high ITT concentration (50- to 100-fold compared to peripheral circulation) is considered necessary for the maintenance of spermatogenesis, and attempts for its suppression have been harnessed for the development of a hormonal male contraceptive (2, 8). If men are treated with testosterone, the heightened negative feedback at the hypothalamic–pituitary level blocks gonadotrophin secretion and the stimulation of testicular testosterone production, rendering the intratesticular milieu unsupportive to spermatogenesis. A major caveat of the testosterone -induced contraception is that only about 60% of Caucasian men (higher in Chinese men) achieve azoospermia or severe oligozoospermia (<1 mill./mL), needed for contraceptive efficacy. No mechanism for the non-uniform spermatogenic suppression has yet been identified. The treatment is more effective if testosterone is combined with progestin to achieve more pronounced gonadotropin suppression (2, 8), but progestins may also have direct inhibitory effects on Leydig cell function (17). The question still remains open how profound the decrease of ITT has to be to maximize the spermatogenic suppression.

Testosterone and spermatogenesis in LuRKO mice

Observations on the LuRKO mouse brought a novel angle to the question of the amount of testosterone needed for the maintenance of spermatogenesis. These mice are born normally masculinized, because testosterone production of mouse fetal Leydig cells, although expressing *Lhr* and responding to LH stimulation, is not dependent on LH action. Numerous non-gonadotrophic hormones and paracrine regulating factors are able to maintain normal fetal Leydig cell steroidogenesis in the absence of LH action (18, 19). In contrast, adult Leydig cell maturation and steroidogenesis in the mouse are possible only with adequate LH stimulation, which explains why the adult LuRKO mice are hypogondal with small cryptorchid testes, azoospermia and ITT <2% of normal (Figs 1 and 2) (15). The progression of spermatogenesis is halted at the round spermatid stage (Fig. 2), in agreement with the known necessity of testosterone to advance it from round to elongating spermatids (1, 3).

Surprisingly, when we examined testicular histology of 12-month-old LuRKO mice, patches of full spermatogenesis with mature spermatozoa were found despite the persistently very low ITT concentration (20). This was in contrast to the absent sperm maturation in young adult LuRKO mice. The question arose therefore whether the spermatogenesis observed in the old LuRKO

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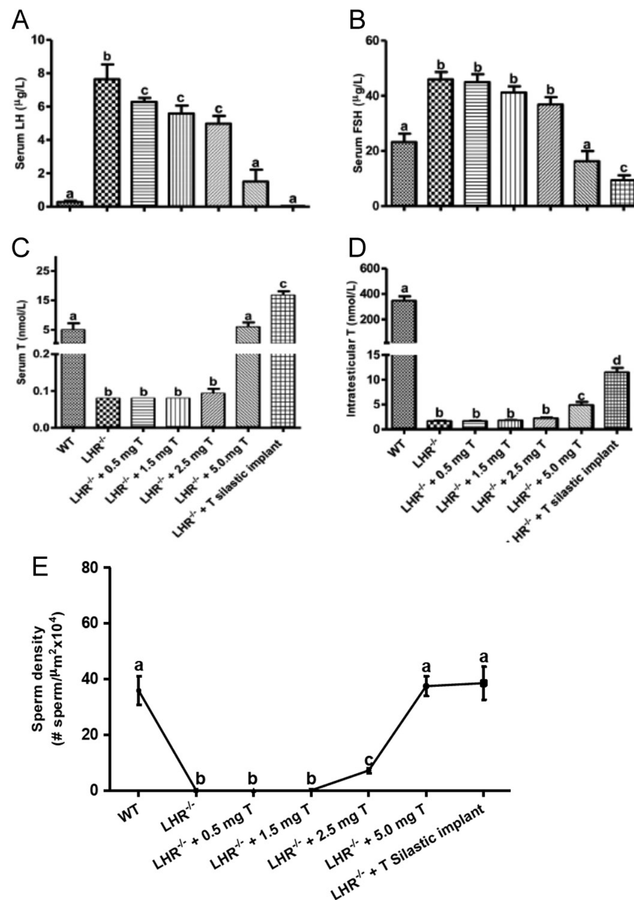

Figure 1

Responses of serum LH (panel A), FSH (B), testosterone (C), intratesticular testosterone (D) and sperm density (E) to increasing doses of testosterone: 0, 0.5, 1.5, 2.5, 5.0 mg/pellet and silastic implant (highest dose) in LuRKO mice. The left bar in each panel depicts the findings in WT mice. The treatment was given between the ages of 30 days and 3 months. It is noteworthy that the same 5 mg dose of testosterone suppressed the gonadotrophins of the LuRKO mice to levels indistinguishable from WT mice, increased serum testosterone to WT level (about 5 nmol/L) and normalized sperm production. These responses were observed when intratesticular (IT) testosterone concentration was the same as in peripheral serum (5 nmol/L), which is only 1.4% on the ITT concentration of WT mice. The figure is modified from the data presented in (21).

mice was maintained by the very low but detectable ITT levels of testosterone (1.5 nmol/L) or the two-fold elevated serum FSH.

To solve the above question, we treated the old LuRKO mice between 9 and 12 mo of age with the antiandrogen flutamide, which completely blocked their sperm maturation at the round spermatid stage. Hence, the low nanomolar concentration of ITT (>95% reduced), and not the 2-fold elevated FSH, was responsible for the qualitatively complete spermatogenesis in the old LuRKO mice. The time needed for this response was considerably prolonged from that occurring in WT mice. The persistent FSH action in these mice alone, when androgen action was blocked, could not compensate for the missing testosterone effect.

Titrating the ITT concentration needed for spermatogenesis

To identify the ITT concentration needed to turn on and maintain spermatogenesis, we treated in the next experiment LuRKO mice with a gradient of testosterone doses, released from subcutaneous pellets or silastic tubing between 3 weeks and 3 months of age (21). A clear dose–response was found, and the effective dose normalizing LuRKO mouse gonadotropins, spermatogenesis and sexual function appeared to be the same 5 mg, and it achieved a circulating and ITT concentration of about 5 nmol/L (Fig. 1). The serum concentration was the same as in WT mice, but the ITT level was less than 1.5% of the WT concentration. Hence, these findings could not recapitulate the dogma that high ITT is needed for the maintenance of spermatogenesis. After all, the banal explanation for the high ITT level could simply be that it is high because the testis is the site of testosterone production.

These findings may have clinical significance for the development of hormonal male contraception. The most promising approach is testosterone treatment, which functions by suppressing gonadotrophins through heightened negative feedback of the administered testosterone on gonadotrophin secretion, resulting in cessation of LH-stimulated testicular testosterone production and its support of spermatogenesis. The main caveat of this approach is that all men treated do not achieve azoospermia, the obvious goal off effective contraception (2, 8). Although not proven, the possible explanation for the insufficient efficacy is that the hiatus between testosterone induced gonadotrophin suppression and direct stimulation of spermatogenesis by the administered testosterone is not wide enough, i.e. as soon as circulating testosterone reaches a concentration suppressing gonadotrophins, it also starts stimulating spermatogenesis. Our findings support this contention, because the same dose of testosterone that brought

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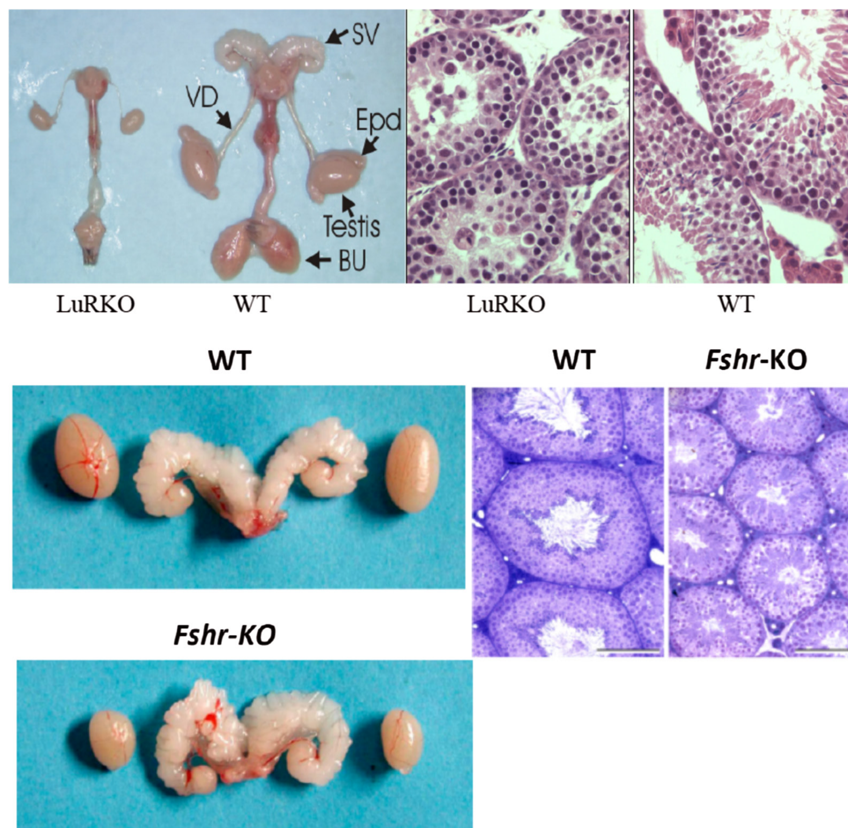

Figure 2
Upper panels: The urogenital blocks of adult *Lhr*-knockout (LuRKO) and WT mice (left panels) and testicular histology of the same genotypes (right panels). All urogenital structures in the LuRKO mice are rudimentary, and their spermatogenesis is interrupted at the round spermatid stage. From (15) with permission. Lower panels: Testes and seminal vesicles of adult WT and *Fshr*-knockout (KO) mice (left panels) and testicular histology of the same genotypes (right panels). While there is no difference in seminal vesicle sized between the two genotypes, the size of the Fshr-KO testes is about half of WT. Although full spermatogenesis is visible in both testes, the tubular diameter is clearly narrower in KO. The figure is a courtesy of Dr H. Charlton (Univ. of Oxford).

about suppression of gonadotrophins also turned on spermatogenesis in the LuRKO mice. Hence, it is apparent that to achieve uniform spermatogenic suppression to azoospermia, more effective inhibition of ITT must be achieved than that attained through the testosterone -induced suppression of gonadotrophins.

Men with inactivating mutations of *FSHR* and *FSHB*

Men with both *FSHR* and *FSHB* mutations have been described. The inactivating C566T point mutation, predicting an A189V substitution of the FSHR protein, belongs to the Finnish heritage of genetic diseases (22), and in 1995, seven families carrying this mutation were identified among women with hypergonadotropic hypogonadism (23). In these families, five homozygous brothers of the women with the syndrome were identified. Curiously, none of the men was azoospermic, which was in contrast to the contention at the time that FSH is needed for the pubertal onset of spermatogenesis. The men had variably reduced testis size and were subfertile with sperm counts varying from oligospermia to normozoospermia.

Subsequently, a total of five isolated cases of inactivating *FSHB* mutations have been described, and curiously, these men are all azoospermic. At least three of the cases were found upon screening of azoospermic men, which may explain why azoospermia was defined as the phenotype of *FSHB* inactivation. The apparent difference between the phenotypes of the FSH hormone and receptor mutations is difficult to explain, because in principle the phenotype of the receptor inactivation should be stronger. Empty WT receptor may have marginal constitutive activity in the absence of functional ligand, but this is not possible if functional receptor is missing. One possible explanation is that the mutant A189V FSHR is not completely inactive. In fact, it has been found that its mechanism of inactivation is intracellular sequestration, and if it is strongly overexpressed *in vitro*, a minute proportion of the receptor reaches the plasma membrane and is able to promote FSH signaling (24). Another curious finding about the *FSHB* mutations is that the affected individuals are totally resistant to FSH treatment (24), which suggests that FSH inactivation may not be the only cause of their azoospermia. The reasons for the difference between the ligand and receptor inactivation phenotypes are likely to remain unclear until more men with inactivating *FSHB* and *FSHR* mutations are identified.

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Knockout mice for *Fshb* and *Fshr*

The KO mice for *Fshb* (11) and *Fshr* (12, 13) have practically identical phenotypes: about 50% reduced testis size, but normal spermatogenesis and fertility (Fig. 2). The phenotype therefore resembles closer that of the human *FSHR* inactivation than that of *FSHB*. On the basis of these phenotypes both KO mouse models support the role of FSH in pubertal proliferation of Sertoli cells, which is important in the determination of the finite testis size. In contrast, its role in the pubertal initiation of spermatogenesis, as proposed earlier (10), is not supported by the KOs. Therefore, at least in the male mouse, FSH is not necessary for spermatogenesis and fertility. In the human, this question still remains open.

Activating *Fshr* mutation maintaining spermatogenesis independent of testosterone

Spermatogenesis is possible without FSH when testosterone exposure is normal, but the opposite has never been documented. To study further the effect of FSH on spermatogenesis, we produced a transgenic mouse that expressed in Sertoli cells, driven by the anti-Müllerian hormone promoter, a strongly activating mutation of *Fshr* (Fshr-CAM) (16). The male mice had no apparent phenotype, which indicates that physiological FSH action brings about maximal FSH effect if all other regulatory mechanisms are normal. Apparently for the same reason, it has been difficult to find activating *FSHR* mutations in men. The only two cases detected have been a hypophysectomized man with normal spermatogenesis (25) and a man with serendipitously detected absence of FSH in the face of normal spermatogenesis (26).

We then crossed the Fshr-CAM and LuRKO mice, in order to create a situation of strong FSHR stimulation in the absence of LH-stimulated testosterone production (27). Unexpectedly, the double-mutant mice had nearly normal spermatogenesis (Fig. 3), and the infertility of LuRKO mice was partially rescued. The testes of the double-mutant mice descended to scrotum, were of normal size, and the seminal vesicles were nearly the size of WT mice, which we considered to be explained by the partial recovery of serum testosterone and ITT, to 40 and 20% of WT, respectively. We therefore ascribed the recovery of spermatogenesis to a rather mundane explanation: the partially recovered Leydig cell testosterone production, apparently through stimulation of Fshr-CAM evoked paracrine factors originating from Sertoli cells.

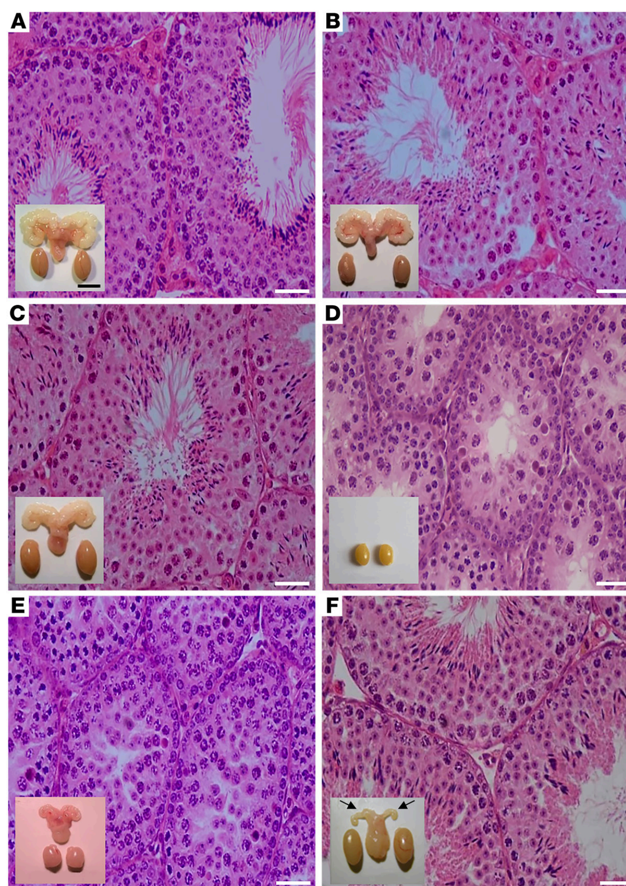

Figure 3
Testicular histology and macroscopic views of testes and urogenital blocks of different mouse genotypes and of flutamide-treated animals: (A) WT, (B) Fshr-CAM, (C) Fshr-CAM/LuRKO, and (D) LuRKO mice. (A, B and C) show normal spermatogenesis and testis and seminal vesicle (SV) sizes. In (D), spermatogenesis is shown as arrested at the RS stage, with small testes and rudimentary SV (not visible). (E) Treatment of WT mice ($n = 5$/group) with The antiandrogen flutamide arrested spermatogenesis at round spermatid stage, with reduced testis and SV sizes. (F) Identical treatment of Fshr-CAM/LuRKO mice had no apparent effect on spermatogenesis and testis size, but reduced SV sizes (arrows). Scale bars: 50 µm; 10 mm (insets). From (27) with permission.

To find out whether the Leydig cell-produced testosterone was accountable for the emerged spermatogenesis in the Fshr-CAM/LuRKO mice, we next eliminated its action by treatment with the strong antiandrogen flutamide. In control WT animals, as expected, seminal vesicles shrank and spermatogenesis

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was halted at the round spermatid stage by flutamide treatment. Quite surprisingly, in the Fshr-CAM/LuRKO mice the antiandrogen had no effect on spermatogenesis (Fig. 3), which meant that, rather than by testosterone, it was maintained by the strong FSH action.

The universal inactivation of androgen action by flutamide in WT mice induced similar azoospermic phenotype as observed in the mice with universal, and Sertoli and peritubular myoid cell-specific *AR* knockouts (28, 29). Persistent spermatogenesis in the flutamide-treated Fshr-CAM/LuRKO mice suggests that strong FSHR activation can compensate for missing AR action, besides Sertoli, also in peritubular myoid cells.

The finding on FSH substituting for testosterone action was unexpected in light of the fact that FSH and testosterone have totally different mechanisms of action, the former through a G-protein-coupled plasma membrane receptor, the latter through a nuclear transcription factor. However, when the signaling cascades triggered by FSHR and AR activation are scrutinized in detail, several overlaps are found (30, 31). Both hormones activate the MAP/ERK and CREB signaling cascades, recently shown to be crucial for spermatogenesis through a rapid testosterone signaling mechanism (32). They also increase Sertoli cell intracellular free Ca^{2+} (33, 34). Thus, testosterone and FSH signaling pathways are really partly overlapping, which explains why strong FSH action is able to compensate for the absence of testosterone in the maintenance of spermatogenesis. The incomplete quantitative recovery of spermatogenesis and fertility in the Fshr-CAM/LuRKO mice, however, emphasizes that qualitatively and quantitatively full spermatogenesis requires testosterone.

Further evidence for the overlap of FSH and testosterone actions was obtained when we ran gene expression arrays of testes of the different genotypes used in our experiments (Fig. 4). Several androgen-dependent Sertoli cell genes, e.g. *Drd4*, *Rhox 5* and *Eppin* (3), demonstrated decreased expression in the flutamide-treated WT and androgen-deprived LuRKO testes. In contrast, flutamide treatment did not reduce their expression in the Fshr-CAM/LuRKO testes, indicating that the strong Fshr-CAM signaling maintained the expression of genes considered strictly androgen regulated. These findings provided the mechanistic explanation for the persistent spermatogenesis in the androgen-deprived and strongly FSH-stimulated testes.

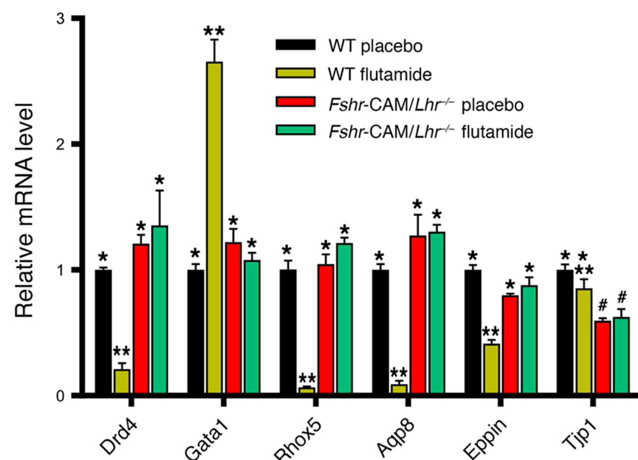

Figure 4
Effect of flutamide treatment on expression of selected androgen-regulated genes in WT and Fshr-CAM/LuRKO ($Lhr^{-/-}$) mice. Data represent mean ± s.e.m., $n = 3$ samples/group. Bars with different symbols differ significantly from each other ($P < 0.05$; ANOVA/Newman–Keuls). The remarkable finding is that while flutamide treatment suppressed the expression strictly androgen-dependent genes (*Drd4*, *Rhox5*, *Aqp8*), the same effect was not observed in the Fshr-CAM/LuRKO testes. From (27) with permission.

Clinical correlates

Our experiments with LuRKO mouse spermatogenesis indicate that the very high ITT concentration is apparently redundant for spermatogenesis, being rather the consequence of the testes being the site of the body's androgen synthesis. This finding augurs difficulties for the development of an effective male contraceptive based solely on testosterone-induced gonadotrophin suppression. The hiatus between the testosterone dose suppressing gonadotrophins and that directly stimulating spermatogenesis appears to be very narrow, if not non-existing, at least in the mouse. The finding calls for novel strategies to suppress spermatogenesis while maintaining simultaneously sufficient peripheral androgen effects (anabolic and sexual functions).

The finding that strong FSH stimulation can replace testosterone in the maintenance of spermatogenesis also has clinical implications. It explains the mechanism of gonadotropin-independent spermatogenesis that has been documented in a hypophysectomized male (25). The patient was hypophysectomized because of acromegaly and was found to have full spermatogenesis to allow fertility in the presence of non-detectable gonadotropins and low, yet higher that post-castration levels of testosterone. Both his spermatogenesis and somewhat increased testosterone

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levels are faithfully phenocopied by the Fshr-CAM/LurKO mouse, thus providing the mechanism for this unusual phenotype.

Our findings may also explain why FSH has proven disappointing in the treatment of idiopathic oligozoospermia (35). The FSH doses have to be higher than the standard 75–150 IU every other day in order to improve spermatogenesis. Indeed, two clinical studies on effects of higher FSH doses on spermatogenesis have been successful (36, 37). In particular, the study of Ding *et al*. (37) showed convincingly a dose-related improvement of spermatogenesis in idiopathic oligozoospermia using doses 2- to 4-fold higher than the usual FSH dose. More extensive clinical studies on this novel treatment strategy are therefore warranted.

Conclusions

To conclude, we have found in our studies on genetically modified mice that the concept of high ITT being necessary for spermatogenesis may not be true and that high FSH stimulation is able to substitute for testosterone in the stimulation of spermatogenesis. Both findings have clinical implications, the former in the development of a male hormonal contraceptive and the latter in the treatment of idiopathic oligozoospermia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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