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Macroorchidism in *FMR1* Knockout Mice Is Caused by Increased Sertoli Cell Proliferation during Testicular Development*

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ABSTRACT

The fragile X syndrome is the most frequent hereditary form of mental retardation. This X-linked disorder is, in most cases, caused by an unstable and expanding trinucleotide CGG repeat located in the 5′-untranslated region of the gene involved, the fragile X mental retardation 1 (*FMR1*) gene. Expansion of the CGG repeat to a length of more than 200 trinucleotides results in silencing of the *FMR1* gene promoter and, thus, in an inactive gene.

The clinical features of male fragile X patients include mental retardation, autistiform behavior, and characteristic facial features. In addition, macroorchidism is observed. To study the role of Sertoli cell proliferation and FSH signal transduction in the occurrence of macroorchidism in fragile X males, we made use of an animal model for the fragile X syndrome, an *Fmr1* knockout mouse.

The results indicate that in male *Fmr1* knockout mice, the rate of Sertoli cell proliferation is increased from embryonic day 12 to 15 days postnatally. The onset and length of the period of Sertoli cell proliferation were not changed compared with those in the control males. Serum levels of FSH, FSH receptor messenger RNA expression, and short term effects of FSH on Sertoli cell function, as measured by down-regulation of FSH receptor messenger RNA, were not changed.

We conclude that macroorchidism in *Fmr1* knockout male mice is caused by an increased rate of Sertoli cell proliferation. This increase does not appear to be the result of a major change in FSH signal transduction in *Fmr1* knockout mice. (*Endocrinology* **139:** 156–162, 1998)

THE FRAGILE X syndrome, an X-linked hereditary disorder, is the most frequent form of inherited mental retardation in humans (see for reviews, Refs. 1 and 2). The gene involved in this disorder, the *FMR1* gene, is located in a region of the X-chromosome, Xq27.3, that cytogenetically displays a fragile site in patients. The *FMR1* gene encodes a cytoplasmic protein with a molecular mass varying from 70–80 kDa, which contains RNA-binding protein motifs (3–5). The fragile X syndrome is caused by the lengthening of a trinucleotide CGG repeat that results in *FMR1* gene silencing (6–9). The repeat is present in the 5′-untranslated region of the *FMR1* gene and shows 6–50 CGG units in the normal population (9). When the repeat length has increased to a length of ~50–200 CGGs, it is called a premutation, as male carriers do not have any symptoms (7, 9, 10). However, female carriers of a premutation show a 4- to 5-fold increase in the incidence of dizygous twinning and increased occurrence of premature menopause (11–13). In the case of a full mutation, when the number of repeats exceeds 200, all male individuals are affected, whereas 50–70% of the females with 1 affected allele express the fragile X clinical phenotype (14–16). In addition to expansion of the CGG repeat, complete or partial deletions of the gene and point mutations have been reported in fragile X patients (17–19). The classical fragile X phenotype in male patients not only includes characteristic facial features and behavioral and learning problems, but also the occurrence of macroorchidism (20–24). In the large testes of fragile X males, no consistent pathological abnormalities have been observed. In general, tubule diameter is mildly reduced, and early spermatogenesis is normal, although malformed spermatids in the later stages of spermatogenesis were identified (25, 26). Testicular enlargement appears to be caused mainly by interstitial edema (27), although in one report it was found that the macroorchidism was caused by a large increase in tubular length (28). Rudelli *et al.* (29) suggested that the increase in testis size in fragile X males initiates prenatally. Thus, the cause of macroorchidism in fragile X males remains unclear.

Recently, a mouse model for fragile X was developed that mimics the gene-silencing effect of the extension of the CGG repeat by inactivation of the *Fmr1* gene through homologous recombination (30). These mice show some characteristics, such as learning difficulties and hyperactivity, that may be related to the features found in fragile X patients. Macroorchidism was also identified in the male *Fmr1* knockout mice. All structural features of the testis in these mice, such as tubule diameter, interstitial cell number, and overall spermatogenesis, appeared to be normal (30). As these observations are at least partially consistent with the macroorchidism found in human fragile X patients, we used the *Fmr1*

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knockout mouse as a model to study the mechanism of the development of macroorchidism.

In the adult, testis size is mainly a reflection of the number of germ cells present. Inhibition of spermatogenesis, *e.g.* by hypophysectomy, results in a spectacular reduction in testis weight. As only a limited number of germ cells can be supported by one Sertoli cell (31), the maximal quantity of germ cells per testis is dependent on the number of Sertoli cells present. Therefore, the increased testis size found in *Fmr1* knockout mice might be a consequence of a higher number of Sertoli cells that causes an increase in tubule length with unchanged tubule diameter. We hypothesized that the increased testis size of adult *Fmr1* knockout mice is the result of an increase in Sertoli cell proliferation, subsequently causing increased germ cell numbers and elevated testis weight.

In the present paper, we report on experiments on Sertoli cell proliferation and FSH signal transduction in the mouse *Fmr1* knockout model. Mitogenic activity of Sertoli cells was determined at different time points during fetal and postnatal life. Because the pituitary hormone FSH is a main determinant of Sertoli cell proliferation and differentiation (31, 32), FSH signal transduction was evaluated through determination of serum FSH concentrations, testicular FSH receptor messenger RNA (mRNA) levels, and short term FSH-induced down-regulation of FSH receptor mRNA *in vivo*. We found that macroorchidism in *Fmr1* knockout mice is caused by increased overall perinatal Sertoli cell proliferation. Changes in FSH signal transduction were not observed, suggesting that the increase in Sertoli cell proliferative activity is not the result of a prominent change in FSH action.

Materials and Methods

Animals and determination of the mouse Fmr1 genotype

The *Fmr1* knockout mice were produced and described by Bakker *et al.* (30). The mutant mice, in which exon 5 of the *Fmr1* gene was inactivated through interruption with a *neo* cassette using homologous recombination, displayed learning deficits and hyperactivity, and an approximately 30% increase in adult testis size.

To analyze the genotype of the mice, genomic DNA was isolated by incubating tissue (tail or fetal head) overnight at 55 C in 300 μl 10 mM Tris-HCl (pH 7.5), 400 mM NaCl, 2 mM EDTA, 66.7 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany), and 1% SDS. The next day, 100 μl 6 M NaCl was added, and the mixture was vortexed vigorously for 15 sec. Subsequently, the solution was centrifuged at maximum speed for 15 min. To the supernatant, 2 vol 100% ethanol were added, and the precipitated DNA was washed with 70% ethanol. After the DNA was air-dried for a few minutes, it was dissolved in 100 μl TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, pH 8), 3 μl of which were used in a PCR reaction.

Screening for the presence or absence of the wild-type allele was performed using primers S1 (5′-GTGGTTAGCTAAAGTGAGGATGAT-3′) and S2 (5′-CAGGTTTGTTGGG-ATTAACAG ATC-3′). Primers M2 (5′-ATCTAGTCATGCTATGGATATCAGC-3′) and N2 (5′-GTGGGC-TCTATGGCTTCTGAGG-3′) were used to screen for the presence of the knockout allele, and primers C8 (5′-ACGAGAAGATCTGATGGGTT-TAGC-3′) and Km4 (5′-GTGGAACCTGTATGACATCTTCA-3′) were used as an internal DNA control. For the PCR, 12.5 pmol of either primers M2 and N2 or primers S1 and S2 were used in a PCR buffer containing 1.2 mM dithiothreitol, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM deoxy-NTPs (Pharmacia, Uppsala, Sweden), 0.5 mM spermidine (Sigma, St. Louis, MO), 0.2 pmol primer C8, 0.2 pmol primer Km4, and 0.2 U Supertaq (SphearoQ, Leiden, The Netherlands) in a total volume of 25 μl.

The PCR reactions were preheated at 94 C for 5 min. Thirty PCR cycles (30-sec denaturation at 94 C, 30-sec annealing at 65 C, 1.5-min extension at 72 C) were performed, followed by an extra extension step of 10 min at 72 C. The products were electrophoresed on a 1.5% agarose gel.

Measurement of serum FSH

Serum from both *Fmr1* knockout and wild type mice was collected. Due to the very low amounts obtained from the smaller animals, sera from two 1-day-old and two 3-day-old mice were pooled. FSH was measured by RIA (33), using antibodies developed by Welschen *et al.* (34). All results are expressed in terms of NIDDK rat FSH RP-3. The intraassay variation was 5.1%, and all samples were measured in one assay.

Sertoli cell labeling index in Fmr1 knockout and wild-type mice

Male mice of different ages were injected ip with 150 μg/g BW 5-bromo-2′-deoxyuridine (BrdU; Boehringer Mannheim). After 2 h, testes were collected, fixed in methacarn [60% (vol/vol) methanol, 30% (vol/vol) chloroform, and 10% (vol/vol) acetic acid] for 1 h or overnight. Subsequently, the fixative was replaced with 70% ethanol, and the tissue was kept in ethanol at 4 C. The testes were embedded in glycol methacrylate (Technovit 7100, Kulzer, Wehrheim, Germany), and 3-μm sections were cut. The sections were processed immunohistochemically using the immunogold-silver staining technique as described by van de Kant and de Rooij (35), except that the second and third antibodies were replaced by biotinylated α mouse IgG and avidin-biotin-peroxidase complex (Elite ABC kit PK 6102, Vector Laboratories, Burlingame, CA), respectively. Finally, the sections were silver stained after incubation with α-horseradish peroxidase-gold (10 nm; Aurion, Wageningen, The Netherlands). The sections were counterstained with Gill's hematoxylin no. 3.

The histological slides were coded, and the percentage of labeled Sertoli cells was determined in a blinded manner, studying 500 Sertoli cells in each animal. In sections of testes from 15-day-old knockout (n = 6) and wild-type (n = 5) mice, the percentage of tubular cross-sections containing spermatocytes was determined by examination of 100 cross-sections of the testis from each animal.

Testicular FSH receptor mRNA expression

Testicular FSH receptor mRNA expression was measured at different ages or at different time points after injection of 15-day-old male mice with 0.15 IU/g BW human FSH (Metrodin, Serono, Geneva, Switzerland). Total testicular RNA was isolated using the LiCl-urea method (36). Mouse FSH receptor antisense RNA probes were generated from a 564-bp *Eco*RV fragment corresponding to bp 1236–1799 of the mouse FSH receptor gene, subcloned in pBluescript KS (pBmFSHR) using T3 RNA polymerase and [^{32}P]UTP. A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe corresponding to bp 197–310 of the rat GAPDH gene (37) was used to determine the relative amount of RNA loaded on the gel. Five or 10 μg total RNA were analyzed by ribonuclease (RNase) protection assay according to the method of Sambrook *et al.* (38).

Statistical analysis

Results are presented as the mean ± SEM. ANOVA and Duncan's new multiple range test were used for statistical evaluation of data. Differences were considered significant at $P \leq 0.05$.

Results

Testicular development in Fmr1 knockout mice

At 15 days of age, *Fmr1* knockout mice have larger testes than their wild-type littermates [weight of two testes in wild-type mice, 17.2 mg ± 0.71 (±SEM; n = 9); in *Fmr1* knockout mice, 21.9 mg ± 1.74 (n = 6); $P < 0.009$]. Younger *Fmr1* knockout animals tended to have larger testis weights, but

these differences were not statistically significant (Fig. 1). As a result of the commencement of the first wave of spermatogenesis, seminiferous tubule diameter doubled between postnatal days 1 and 15 (results not shown), but no difference between control and *Fmr1* knockout mice could be discerned. There was no difference in the general testicular morphology between the two groups.

The progress of the first wave of spermatogenesis was determined by the percentage of tubular cross-sections showing spermatocytes on day 15 after birth. In *Fmr1* knockout mice and wild-type mice, 72 ± 12 and 70 ± 12 (mean ± SEM) of the cross-sections contained spermatocytes. The most advanced type of spermatogenic cells present at 15 days varied considerably, but ranged from early to midpachytene spermatocytes in both the wild-type and knockout mice (Fig. 2), indicating that the increase in testis size in *Fmr1* knockout mice is not caused by an earlier onset of spermatogenesis.

Mitotic activity of testicular Sertoli cells

The proliferative activity of Sertoli cells in the developing testis of *Fmr1* knockout mice was compared with that in wild-type littermates to determine whether the increase in testis weight was caused by increased mitogenic activity of the Sertoli cells or a prolonged period of Sertoli cell proliferation. Mice of different ages were injected ip with BrdU, and their testes were isolated after 2 h. Subsequently, the labeling index of Sertoli cells was determined as a measure of the proliferative activity of these cells. As depicted in Fig. 3, the overall pattern of Sertoli cell proliferation is similar in mice of both genotypes, *i.e.* the maximal labeling index is found on day 17 postcoitum, whereas Sertoli cell proliferation comes to a stop on postnatal day 15. Thus, it appears that the developmental period during which of Sertoli cell divisions normally occur does not change in the *Fmr1* knockout mice. However, as can be clearly seen in Fig. 3, overall comparison of the values for *Fmr1* knockout mice and wild-type mice at all time points revealed a significant increase of 19.4 ± 4.8% ($P = 0.0003$, by ANOVA). The labeling index values at embryonic day 15 (E15) and E17 are significantly different between wild-type and *Fmr1* knockout mice ($P \leq 0.05$, by Duncan's new multiple range test).

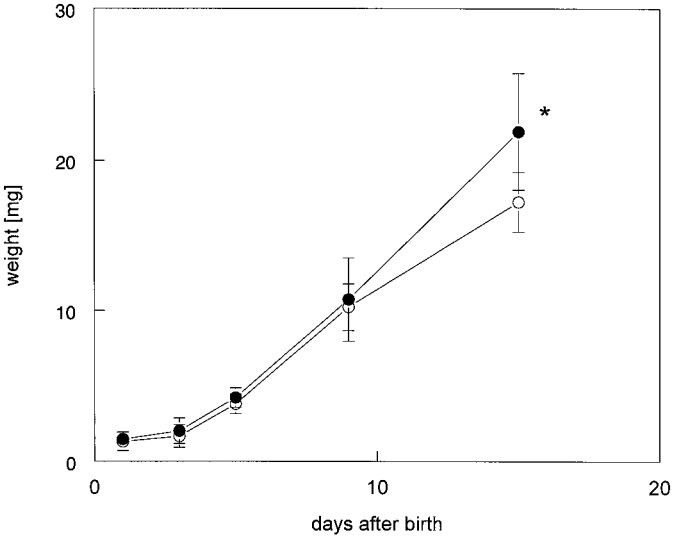

FIG. 1. Testis weight in *Fmr1* knockout mice (●) and wild-type littermates (○). At 15 days of age, a significant increase in testis weight was observed ($P < 0.009$). The combined weight of both testes is given. The numbers of animals in each group were (wild-type and *Fmr1* knockout mice, respectively): day 1, 9 and 9; day 3, 3 and 9; day 5, 7 and 5; day 9, 3 and 7; and day 15, 7 and 4. Data represent the mean ± SEM (n = 6–9).

Evaluation of FSH signal transduction

A main determinant of Sertoli cell proliferation and differentiation is the glycoprotein hormone FSH, which is produced in the pituitary gland. We evaluated different components of the FSH signal transduction pathway to determine whether FSH might be involved in the change in testis weight in *Fmr1* knockout mice. Serum levels of FSH were determined in trunk blood from mice at different ages. The FSH serum concentration of control animals increased from 2.3 ng/ml on day 1 to 18.6 ng/ml on day 15, and there was no statistically significant difference from the values in the *Fmr1* knockout mice (Fig. 4).

Another parameter that influences FSH signal transduction is the level of FSH receptor mRNA expression in the Sertoli cells. FSH receptor mRNA levels and GAPDH mRNA as a measure of the amount of RNA in the assay were determined using the RNase protection assay (Fig. 5). Total testicular RNA was used, because the FSH receptor is present in Sertoli cells only. FSH receptor mRNA expression in both wild-type and *Fmr1* knockout animals was maximal on postnatal day 1 and decreased to approximately 50% on day 15. A slight, but not statistically significant, increase in the level of FSH receptor mRNA expression was observed in the *Fmr1* knockout mice (Fig. 5), probably as a result of an increased number of Sertoli cells in the testis.

Although no major changes in serum FSH levels and FSH receptor mRNA were observed, the effectiveness of FSH signal transduction might be changed in the *Fmr1* knockout Sertoli cells. Previously, we have shown that the FSH receptor mRNA level in immature rat testes is down-regulated very rapidly by FSH through the cAMP pathway due to destabilization of the FSH receptor mRNA (39). The *Fmr1* protein might affect the efficiency of coupling by an as yet unknown mechanism or exert a more direct effect by changing FSH receptor mRNA stability by binding to the FSH receptor message with one or more of its RNA-binding regions (3, 5). Mice were injected at the age of 15 days with human FSH, and testicular FSH receptor mRNA levels were determined at different time points after the injection (Fig. 6). It was observed that also in mice, FSH down-regulates its cognate receptor mRNA very rapidly to 30% of control levels. However, neither the kinetics nor the measure of this effect were changed in the absence of the *Fmr1* protein.

Discussion

Testicular size is mainly determined by the number of Sertoli cells that support the proliferation and differentiation of a species-dependent maximum number of germ cells (31). We limited our study of Sertoli cell proliferation in *Fmr1* knockout mice to the last period of fetal development and the first 2 weeks after birth, because a population of identifiable

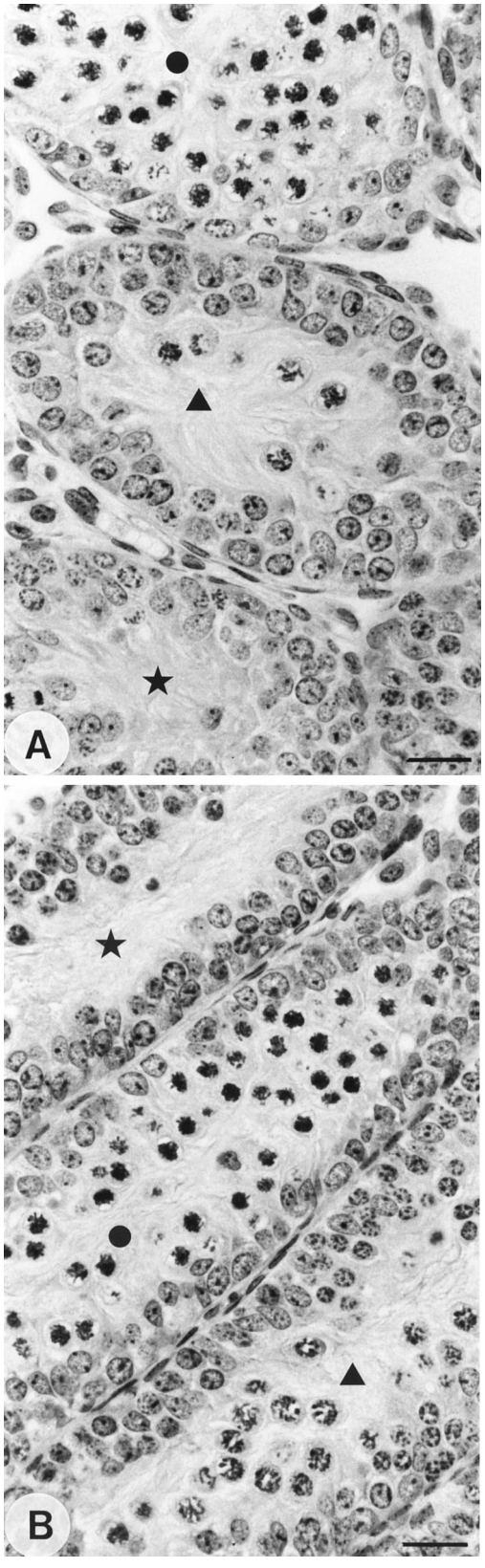

FIG. 2. Spermatogenesis in 15-day-old wild-type (A) and *Fmr1* knockout (B) mice is quite comparable. *, Tubules in which spermatogenesis has proceeded up to spermatogonia; ●, tubules with early pachytene spermatocytes; ▲, tubules with midpachytene spermatocytes. *Bar* = 20 μm; magnification, ×500.

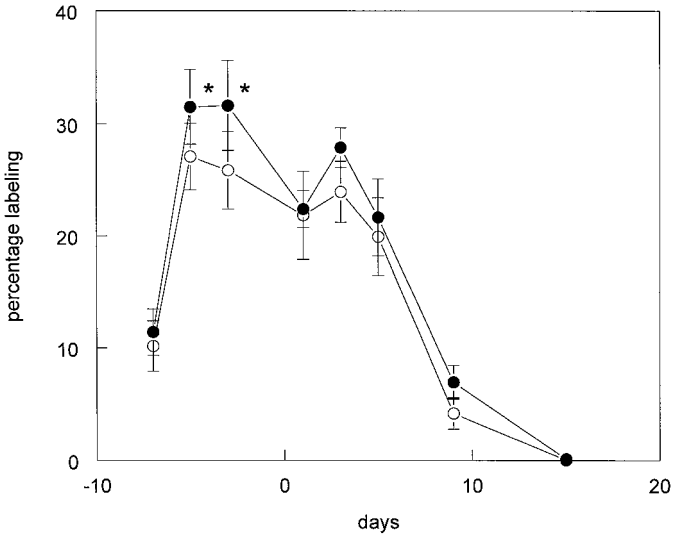

FIG. 3. Sertoli cell labeling index in testes of *Fmr1* knockout mice (●) and wild-type mice (○; $P = 0.0003$). Two hours after an ip injection of BrdU, testes of mice of different ages were collected, and the BrdU labeling index of the Sertoli cells was determined. The numbers of animals in each group were (wild-type and *Fmr1* knockout mice, respectively): day −7, 3 and 8; day −5, 7 and 4; day −3, 12 and 6; day 1, 7 and 6; day 3, 4 and 6; day 5, 6 and 6; day 9, 3 and 5; and day 15, 3 and 4. Data represent the mean ± SEM. Overall comparison of the values of *Fmr1* knockout mice and wild-type mice at all time points revealed a significant increase of 19.4 ± 4.8% ($P = 0.0003$, by ANOVA). The *asterisks* indicate significant differences between *Fmr1* knockout mice and wild-type mice on days −5 and −3 ($P \leq 0.05$, by Duncan's new multiple range test).

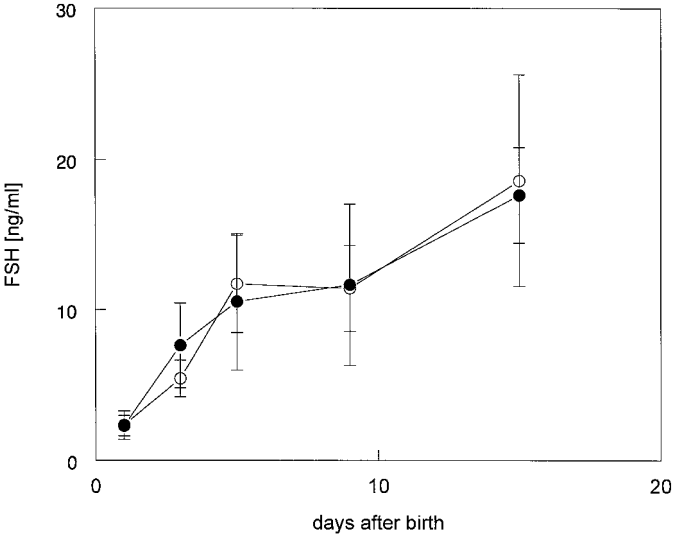

FIG. 4. Serum FSH levels in *Fmr1* knockout mice (●) and wild-type littermates (○). The numbers of animals in each group were (wild-type and *Fmr1* knockout mice, respectively): day 1, 4 and 8; day 3, 3 and 8; day 5, 7 and 4; day 9, 7 and 8; and day 15, 9 and 5. Data represent the mean ± SEM

Sertoli cells is present from E12, and the Sertoli cells have stopped dividing on postnatal day 15 (40, 41).

The magnitude of Sertoli cell proliferative activity is strongly regulated by FSH, whereas the end of the period of mitotic activity appears to be controlled by thyroid hormone. Exogenous administration of FSH to immature rats or pri-

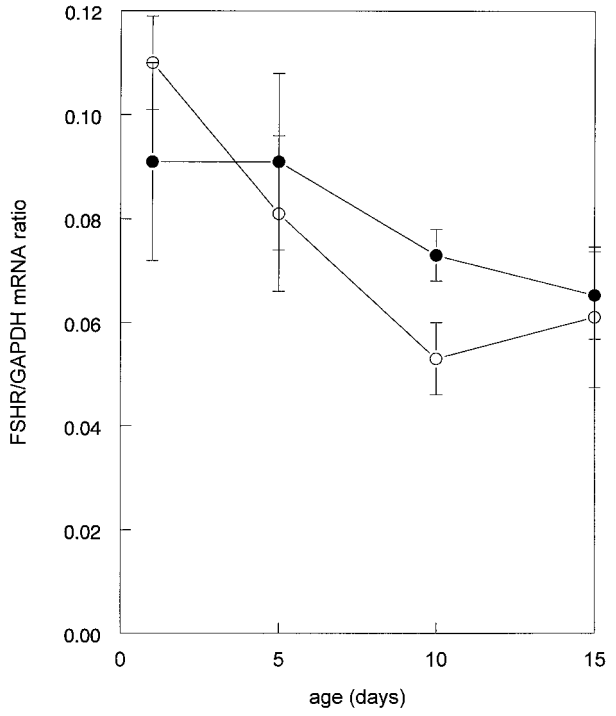

FIG. 5. FSH receptor mRNA expression in *Fmr1* knockout mice (●) and wild-type mice (○) of different ages. Total testicular RNA was isolated and subjected to RNase protection assay with FSH receptor and GAPDH probes. Subsequently, the FSH receptor/GAPDH mRNA ratio was determined. Data represent the mean ± range (n = 2).

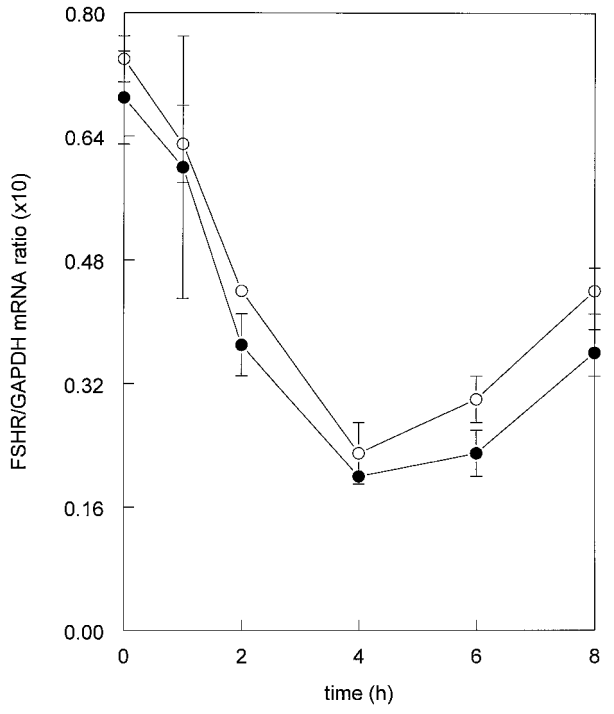

FIG. 6. FSH receptor mRNA down-regulation in *Fmr1* knockout (●) and wild-type mice (○). *Fmr1* knockout male mice and wild-type littermates were injected (ip) with metrodin and killed at different time points after injection. Total testicular RNA was isolated and subjected to RNase protection assay with FSH receptor and GAPDH probes. Subsequently, the FSH receptor/GAPDH mRNA ratio was determined. Data represent the mean ± range (n = 2).

mates or addition of FSH to cultured Sertoli cells increases the mitotic activity of Sertoli cells (42–45). Also, hemicastration of immature rats has been demonstrated to increase the mitotic activity of Sertoli cells in the remaining testis. The mechanism underlying this observation is found within the hypothalamic-pituitary-testicular axis. After unilateral castration, production of inhibin by the Sertoli cells decreases, resulting in less negative feedback action on the pituitary and an increased FSH level that stimulates the Sertoli cells to divide (46–48). Decapitation of fetuses *in utero* to eliminate the fetal hypothalamo-pituitary system or the reduction of FSH in fetuses by administration of FSH antibodies also affects Sertoli cell function during fetal development (49). FSH binding to fetal rat testis is observed on day 17.5 postcoitum and is increased significantly by 20.5 and 21.5 days of fetal life (50). Furthermore, Orth *et al.* (41), showed that during these days of fetal life, the rate of Sertoli cell proliferation in rats is maximal and declines thereafter, suggesting that the Sertoli cells are probably most sensitive to the mitogenic activity of FSH at the end of the fetal period and shortly after birth. The important, but not essential, role of FSH in Sertoli cell proliferation was recently demonstrated in a FSHβ knockout mouse, which showed more than a 50% decrease in adult testis weight (51). This decrease could not be explained by impaired spermatogenesis alone, because the FSHβ knockout males were fertile and showed only a slight decrease in tubule diameter, with normal appearing Sertoli cells and seminiferous epithelium.

Hypothyroidism, induced by the administration of 6-propyl-2-thiouracil to rats, leads to retardation of Sertoli cell differentiation and an extended period of Sertoli cell proliferation, resulting in an increased number of Sertoli cells and macroorchidism (52–54). In contrast, a shorter period of Sertoli cell division and faster differentiation of Sertoli cells are found both *in vitro* in the presence of thyroid hormone and *in vivo* in hyperthyroid animals (55, 56). An effect of thyroid hormone on the FSH level *in vivo* could be excluded because a slight decrease in serum FSH was found after both treatments.

From our studies of Sertoli cell proliferation in the *Fmr1* knockout mice, it appeared that the period of Sertoli cell proliferation had not changed, but, rather, the proliferative activity of the Sertoli cells in *Fmr1* knockout mice was significantly higher, resulting in an increase in germ cell number and testicular weight. These results indicate that the thyroid hormone status of the *Fmr1* knockout mice is not changed and prompted us to investigate several elements of the FSH signal transduction pathway.

The circulating FSH levels in *Fmr1* knockout mice were not elevated compared with those in wild-type littermates. Similarly, there is no evidence for increased FSH in fragile X humans (57, 58). The level of FSH receptor mRNA in the testis of *Fmr1* knockout mice was slightly, but not significantly, higher compared with the wild-type level. The increased number of Sertoli cells in the testes of *Fmr1* knockout mice could have caused this, although other possibilities, such as a change in RNA stability, cannot be ruled out. The RNA-binding activity of FMRP could play a role in the regulation

of FSH receptor mRNA stability, but the FSH-induced down-regulation of FSH receptor mRNA, which is caused by a decrease in receptor mRNA stability (39), was not different in *Fmr1* knockout mice.

We conclude that a change in circulating FSH levels or signal transduction through the FSH receptor is not involved in the increase in testis size in *Fmr1* knockout mice. The testicular localization of the *Fmr1* protein (FMRP) and mRNA is still not clear, and the primary cause of the increased testis size in *Fmr1* knockout mice may be found in either Sertoli cells or germ cells. Recently, it has been reported that contraction of the full mutation to premutation values (<200) occurs in germ cells of human fragile X male fetuses that carry the full mutation in their somatic cells (59). These observations indicate that expression of FMRP in germ cells in an environment of somatic cells that lack FMRP protein still results in macroorchidism, thus diminishing the potential role of germ cells in macroorchidism.

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