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Altered Structure and Function of Reproductive Organs in Transgenic Male Mice Overexpressing Human Aromatase*

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ABSTRACT

Aromatization of androgens is a key step in estrogen production, and it regulates the delicate balance between estrogens and androgens in the gonads and sex steroid target tissues. In the present study, we generated transgenic mice ($AROM^+$) bearing the human ubiquitin C promoter/human P450 aromatase fusion gene. $AROM^+$ male mice are characterized by an imbalance in sex hormone metabolism, resulting in elevated serum E_2 concentrations, combined with significantly reduced testosterone and FSH levels, and elevated levels of PRL and corticosterone. $AROM^+$ males present a multitude of severe structural and functional alterations in the reproductive organs, such as cryptochidism associated with Leydig cell hyperplasia, dysmorphic seminiferous tubules, and disrupted spermatogenesis. The males also have small or rudimentary accessory sex glands with abnormal morphology; a prominent prostatic utricle with squamous epithelial metaplasia, and edema in the ejaculatory ducts and vas deferens. In addition, the abdominal muscle wall is thin, and the adrenal glands are enlarged, with cortical hyperplasia. Some of the abnormalities, such as undescended testes and undeveloped prostate, resemble those observed in animals exposed perinatally to high levels of exogenous estrogen, indicating that the elevated aromatase activity results in excessive estrogen exposure during early phases of development. Some of the disorders in the reproductive organs, furthermore, can be explained by the fact that $AROM^+$ males are hypoandrogenic, and have elevated levels of serum PRL and corticosterone. Thus, the $AROM^+$ mouse model provides a novel tool to investigate the consequences of a prolonged increase in conversion of androgens to estrogens which results in complex hormonal disturbances altering the structure and function of various male reproductive organs. (*Endocrinology* **142:** 2435–2442, 2001)

THE ROLE OF androgens in the development of male reproductive tissues and reproductive performance is well characterized, but the role of estrogens in male reproduction remains to be elucidated further. Estrogens are known to be involved in the negative feedback regulation of gonadotropin secretion in men (1, 2), and are important for the masculinization of the male brain during development and for the maintenance of sexual behavior during adulthood (3). The two forms of estrogen receptors (ER), ERα and ERβ, have been shown to be widely distributed in male reproductive organs, including the testis, efferent ducts, epididymis, vas deferens, bulbo-urethral glands, prostate, and seminal vesicles (4–6). This suggests a direct role for estrogens in male urogenital systems. Recently, the generation of knockout mice as regards ERα (7), ERβ (8) and P450 aromatase (P450arom; Ref. 9) have expanded our understanding of estrogen action, and provided new insights into the role of estrogens in the male. For example, ERα-deficient male mice are infertile, presenting reduced mating frequency and low sperm number with abnormal testicular morphology (10). Studies on ERα-deficient male mice further showed that the males have a failure in the reabsorption of seminiferous tubule fluid in the efferent ductules, resulting in a defect in posttesticular sperm maturation, leading to infertility (11). Male mice deficient in P450arom are initially fertile but show disrupted spermatogenesis and infertility at an older age (12). Furthermore, ER$\alpha^{-/-}$ males, but not ER$\beta^{-/-}$ males, exhibit abnormal sexual behavior (13, 14). Therefore, the data show an essential role, direct or indirect, for estrogens in male reproductive functions.

The P450arom enzyme is the product of the *cyp19* gene (15) and it catalyzes aromatization of the A-ring of androgens such as testosterone (T) and androstenedione, resulting in the formation of a phenolic A ring characteristic of estrogens, E_2 and estrone, respectively (16, 17). Aromatization of androgens by P450arom is one of the final steps in ovarian E_2 biosynthesis, but the enzyme is also widely expressed in female and male extragonadal tissues. However, extragonadal tissues lack the capacity to synthesize androgenic precursors, and estrogen production is dependent on these precursors produced in the gonads. Interestingly, androgen receptor, ERβ and ERα, as well as P450arom, are colocalized in several tissues of male reproductive organs, often with a cell-specific expression pattern in the tissues (6, 18). This suggests that there is a delicate balance between estrogen and androgen action in male reproductive organs. Most impor-

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tantly, several studies on both humans and rodents indicate that prenatal or early postnatal exposure to exogenous estrogens induce severe persistent changes in the structure and function of male reproductive organs, such as atrophic and small testes, epididymal cysts, and abnormalities in the rete testis (19–21). The results of some studies also suggest that estrogens might have a central role in the mechanisms leading to male reproductive tract malformations such as cryptorchidism and enlarged prostatic utricle, and diseases such as testicular (21, 22) and prostatic tumors (23).

To study further the role of estrogen/androgen balance in male reproductive functions, we have generated a transgenic mouse model with an imbalance in sex hormone metabolism by expressing P450arom under the human ubiquitin C promoter. The promoter is known to be activated on embryonic day 15 in mice (24), and ubiquitous and permanent transgene expression in a large variety of tissues is expected. Hence, in this mouse model, there is increased conversion of androgens to estrogens at all phases of testicular androgen production, in perinatal life, during puberty and in adults. Thus, the mouse lines developed provide a novel model to study the physiological consequences of estrogenization of males during various phases of their life span.

Materials and Methods

Construction of the transgene

An expression vector for human P450arom (pUbC-AROM) was constructed by using the pRC/CMV plasmid (Stratagene, La Jolla, CA) as a backbone. A 2.4-kb *Hin*dIII/*Xba*I fragment coding for full-length human P450arom complementary DNA (cDNA; provided by Prof. Evan Simpson, Prince Henry's Institute of Medical Reseach, Clayton, Australia) was subcloned into the multiple cloning site of the pRC/CMV plasmid. The CMV promoter of the vector was then replaced with a 1.0-kb-long ubiquitin C promoter (*Bgl*II/*Hin*dIII fragment, provided by Prof. Peter Angel, Deutsches Krebsforschungszentrum Heidelberg, Heidelberg, Germany). To confirm the activity of the pUbC-AROM expression vector, stable cell lines with an increased P450arom activity were generated in HEK-293 cells.

The 4.0-kb-long pUbC-AROM fragment was released from the vector backbone by digestion with *Bgl*II and *Dra*I enzymes. Before microinjection, the fragment was resolved in 1% agarose gel and isolated by electroelution followed by purification by means of Elutip-D columns (Schleicher & Schuell, Inc., Keene, NH). Finally, the fragment was diluted in TE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 7.5) at a concentration of 2 ng/μl.

AROM$^+$ transgenic founder mice

Using the purified pUbC-AROM expression vector (Fig. 1A), transgenic mice were produced by a standard technique. The pronuclei of fertilized eggs from the FVB/N strain were microinjected with the DNA (2 ng/μl). Microinjected oocytes were implanted into oviducts of pseudopregnant female mice (NMRI strain) and carried to term. Positive founders for the transgene (AROM$^+$) were identified by Southern blot analysis of DNA obtained from tail biopsies. Genomic DNA (10 μg) was digested with *Eco*RI and resolved by electrophoresis in 0.8% agarose gel. The DNA was then blotted onto nylon membrane, the membrane was cross-linked by UV, prehybridized for 1 h at 64 C in hybridization buffer (5 × SSC, 5× Denhardt's solution, 0.5% wt/vol SDS), and with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Aylesbury, UK) -labeled P450arom cDNA overnight at 64 C in the hybridization buffer. The membranes were then washed in 2× SSC, 0.1% SDS once at 64 C for 15 min, once in 0.5× SSC, 0.1% SDS at 64 C for 15 min, and then exposed to x-ray films for 1–3 days at −80 C.

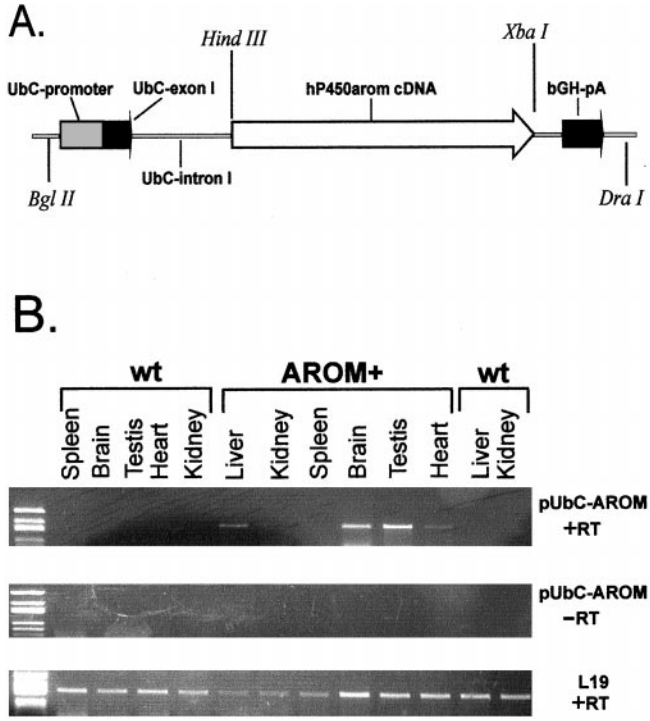

FIG. 1. A, Schematic representation of the pUbC-AROM transgene construct. The transgene consists of a 1.0-kb human ubiquitin C promoter fragment fused with a 2.4-kb-long fragment of human P450arom cDNA coding for the full-length protein, and a 3′-untranslated region and polyadenylation signal of bovine GH. B, RT-PCR analysis of the pUbC-AROM transgene expression in 4-month-old AROM$^+$ male mice (line 21). The transgene was detected both in testis and extragonadal tissues (brain, heart, and liver).

Establishment of AROM$^+$ transgenic lines

Two of the five AROM$^+$ founder mice generated (one male, no. 33, and one female, no. 21) were fertile and they were used to produce subsequent generations by breeding with the wild-type (WT) FVB/N mouse background. All the male mice born of both lines (from the F_1 generation and thereafter) were infertile; hence, the transgenic lines could be established only by mating AROM$^+$ females with WT FVB/N males. The phenotypic characteristics of the AROM$^+$ male mice shown here were obtained using F_1 to F_4 generations of these crossings. As the negative fetuses of these transgenic females were also potentially exposed to extra doses of estrogens during fetal life, they were not used as negative controls. Instead, animals of WT FVB/N breeding pairs were used for this purpose. The mice were housed one to six per cage in controlled conditions of light and temperature. They were fed with commercial mouse chow and tap water *ad libitum*. All mice were handled in accordance with the institutional animal care policies of the University of Turku (Turku, Finland).

For routine genotyping of the AROM$^+$ mice, PCR analyses were carried out using DNA extracted from tail biopsies. The sequences of the PCR primers were as follows: 5′-TGGCGAGTGTGTTTTGTG-3′ (forward primer) and 5′-CCATCTTGTGTTCCTTGACC- 3′ (reverse primer). The PCR (30 cycles) were carried out in a 50-μl volume using the following program: denaturation at 94 C for 1 min, annealing at 50 C for 1 min, and extension at 72 C for 1 min. The resulting PCR products were analyzed by electrophoresis on 2.0% agarose gel and the 200-bp-long PCR products were visualized with ethidium bromide.

Identification of transgene expression by RT-PCR

Total RNA was isolated from brain, heart, kidney, liver, spleen, and testis using the acid phenol method, and RT-PCR was carried out. Four micrograms of total RNA were incubated with 10 IU of avian myeloblastosis virus reverse transcriptase (Finnzymes, Espoo, Finland) at 50

C for 20 min. The cDNAs were then denatured at 95 C for 5 min and amplified by 25 cycles of PCR using the following conditions: 94 C for 1 min, 50 C for 1 min, and 72 C for 1 min. The primers used were as follows: 5′-ACCTCTACACCGCTCTTC-3′ (forward primer) and 5′-GCTTCTCTCACCAATAACAGTC-3 (reverse primer). As an internal control, a 200-bp fragment of the L19 ribosomal protein gene was amplified, using 5′-GAAATCGCCAATGCCAACT-3′ (forward primer) and 5-TCTTAGACCTGCGAGCCTCA-3′ (reverse primer). An aliquot of the RT-PCR product was subjected to agarose gel electrophoresis and visualized by ethidium bromide staining.

Measurement of serum hormones and morphological and histological analyses

WT and AROM$^+$ mice were obtained at similar ages. They were anesthetized by ip injection of 300–600 μl 2.5% avertin. Blood was collected by cardiac puncture, and tissues were dissected out for macroscopic analyses and for obtaining the organ weights. Serum samples were separated by centrifugation and stored at −20 C until hormone concentrations were measured. Concentrations of LH and FSH were measured by using time-resolved immunofluorometric assays (25, 26). Serum T was measured by RIA after diethyl ether extraction, as described previously (27). Concentrations of E_2 were measured using a commercial RIA kit (Immunotech, Beckman Coulter, Inc., Marseille, France), according to the manufacturer's instructions. For histological evaluation, the tissues were fixed in 4% paraformaldehyde, except for the testes, which were fixed in Bouin's solution. The tissues were then dehydrated, embedded in paraffin, and sectioned. Two- to three-micrometer-thick sections were deparaffinized in xylene and then stained with hematoxylin and eosin.

Statistical analysis

Statview software (Statview for Windows, v. 4.57; Abacus Corporation, Berkeley, CA) was used for ANOVA and Fisher's Protected least significient difference *post hoc* tests.

Results

In the present study, transgenic mice expressing human P450arom cDNA under control of the ubiquitin C promoter (Fig. 1A) were generated. A total of five transgenic founder mice (two males and three females) were identified by Southern hybridization analysis of genomic DNA obtained from tail biopsies. Three of them (two females and one male) were infertile as judged by their inability to produce offspring over a 4-month period. This suggests that a high overexpression of P450arom may disrupt the reproductive function both in males and females.

The two fertile founders (one female, no. 21, and one male, no. 33) transmitted the transgene to subsequent generations in a Mendelian fashion, and were used to produce AROM$^+$ transgenic lines. To analyze the distribution of transgene messenger RNA (mRNA) expression in AROM$^+$ mice, RT-PCR was carried out on mRNA prepared from a variety of tissues in the line 21. The strongest signal for transgene mRNA was detected in the testis, and the mRNA was detected at a lower level in the heart, brain, and liver (Fig. 1B). Hence, it is evident that the transgene is expressed both in gonadal and extra-gonadal tissues of the AROM$^+$ male mice.

To analyze the consequences of the transgene expression into circulating hormone concentrations, serum sex steroid and gonadotropin concentrations were measured in 4-month-old AROM$^+$ mice of the line 21, and were compared with WT mice. The AROM$^+$ male mice demonstrated elevated E_2 levels of 98–225 pg/ml (Fig. 2A), whereas in the WT males E_2 concentrations were below the detection limit of the assay used (10 pg/ml). In accordance with the high E_2 concentration, serum T concentrations in the AROM$^+$ males were dramatically reduced (Fig. 2B). However, detectable T concentrations were present (65–323 pg/ml), showing that T biosynthesis continued, providing an androgenic precursor for E_2 biosynthesis in the AROM$^+$ males. Because sex ste-

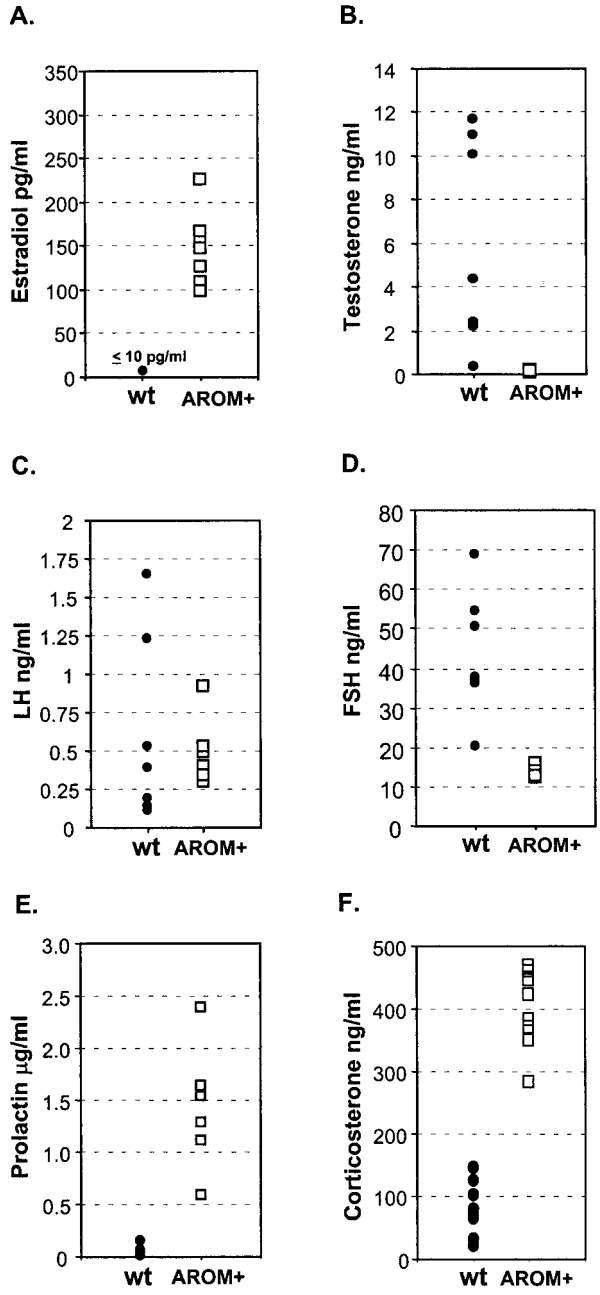

FIG. 2. Serum hormone concentrations in AROM$^+$ male mice (line 21) at the age of 4 months. A, E_2 concentrations in the AROM$^+$ males were between 98–225 pg/ml, whereas, in the WT male mice, no E_2 was found above the limit of detection (10 pg/ml) of the method used. B, T concentrations in AROM$^+$ males were clearly reduced compared with those in WT mice but they were still measurable. LH concentrations in the AROM$^+$ males were within the normal range (C), whereas FSH values were significantly reduced (D). PRL (E) and corticosterone (F) concentrations in AROM$^+$ mice were also markedly elevated as compared with the WT mice. n = 6–10 mice in all groups.

roids are known to exert strong feedback effects on serum gonadotropins, we also measured the circulating concentrations of LH and FSH. No significant difference was found between LH levels in AROM$^+$ and WT males (Fig. 2C). However, there was less variation in the serum LH values in the AROM$^+$ males, suggesting that their LH secretion probably displayed less marked pulsatility. Interestingly, the mean serum FSH levels were moderately decreased in the AROM$^+$ males, compared with age-matched WT mice (Fig. 2D). In addition to the sex hormone imbalance, highly elevated levels in serum PRL (Fig. 2E) and corticosterone (Fig. 2F) concentrations were measured in 4-month-old AROM$^+$ males.

Continuous mating was carried out to analyze the fertility of the AROM$^+$ females and males up to 6 months of age. All the AROM$^+$ males of the F_1 generation and thereafter failed to have offspring. It was unknown why all of the males from the F_1 generation from the mouse line generated from a fertile founder male (no. 33) were infertile. However, it is known that the transgene expression in a mouse line may vary in the first generations; hence, the phenotype is also often unstable during the first generations. To determine the cause of the infertility in AROM$^+$ males, morphological analysis of the reproductive organs was carried out in both AROM$^+$ lines (21 and 33), and multiple abnormalities were observed. The phenotypes were similar in both of the mouse lines and were consistent in all the mice analyzed (summarized in Table 1). In contrast to males, the AROM$^+$ females were not found to have drastic defects in their reproductive functions. They went through pregnancy, delivered pups, and nursed the offspring. However, the possible long-term effects of elevated P450arom expression in the female mice remain to be analyzed.

Southern blot analysis indicated that the highest copy number was present in founder number 31. The infertile male founder showed no signs of reproductive tract dysfunction until the age of puberty. At that time it became evident that the external genitalia of the mouse were not developed normally, and a severe inguinal hernia was observed (Fig. 3A). Furthermore, the testes were enlarged, with Leydig cell hypertrophy/hyperplasia. However, the tubular structures appeared normal in light microscopy, and thus the reason for infertility is not known.

All the AROM$^+$ males (F_1 to F_4 generations) were cryptorchid, with the testes located in the bottom of the abdominal cavity (Fig. 3B), and the weights of the testes, epididymis, seminal vesicles, and prostate lobes were significantly reduced (Fig. 3C and Table 1). In the seminiferous epithelium of the testis, there were no germ cells beyond the stage of pachytene, and numerous degenerating germ cells could be seen near the lumen (Fig. 4, B and C). Furthermore, cells with intensively staining nuclei and eosinophilic cytoplasm, morphologically resembling eosinophil leukocytes, were detected in seminiferous tubules (Fig. 4, C and E). In addition, numerous vacuoles of different sizes were observed within the seminiferous epithelium (Fig. 4, B and C). The interstitium was enlarged and filled with two populations of cells, namely hypertrophic Leydig cells and large multinucleated cells (Fig. 4, C and D). The Leydig cells displayed swollen cytoplasm and ocular nuclei, and appeared to be larger than those of the WT males.

As another steroidogenic organ, we analyzed the weights of adrenal glands of 4-month-old male mice, and they were significantly greater than those of control mice at the same age (Table 1). In line with the increased corticosterone production, histological examination showed adrenocortical hyperplasia (Fig. 4, G and I), with pronounced expansion of the innermost cortical layer, apparently the X-zone, filled with large centripetal vacuole-filled structures (Fig. 4I).

Histological examination further confirmed the small seminal vesicles and prostate glands (Fig. 3) with undifferentiated stratified epithelium and uncanalized bud-like formations surrounded by dense fibromuscular stroma (Fig. 5B). In the collecting ducts, squamous epithelial metaplasia (Fig. 5C) was present in all AROM$^+$ males analyzed, although the extent varied from animal to animal. In some AROM$^+$ males a prominent prostatic utricle with keratinized stratified squa-

TABLE 1. Reproductive phenotypes in AROM$^+$ male mice

Phenotype	Line 21	Line 33	FVB/N control
Infertility	Yes	Yes	
Cryptorchidism	Yes	Yes	
Testis size	Reduced	Reduced	
Testis histology	Leydig cell hyperplasia Abnormal seminiferous tubules	Leydig cell hyperplasia Abnormal seminiferous tubules	
Epididymis	Reduced	Reduced	
Prostate size	Reduced	Rudimentary	
Seminal vesicle size	Rudimentary	Rudimentary	
Adrenal gland size	Enlarged	Enlarged	
Adrenal gland histology	Cortical hyperplasia	Cortical hyperplasia	
Abdominal muscle layer	Thin	Thin	
Pituitary gland	Enlarged	Enlarged	
Mammary gland	Gyneacomastia	Gyneacomastia	
Organ weights			
Testis	47.0 ± 11.34 mg[a]	57.3 ± 16.70 mg[a]	102.7 ± 12.65 mg
Epididymis	23.5 ± 5.85 mg[a]	24.5 ± 6.12 mg[a]	46.2 ± 7.48 mg
Adrenal gland	6.59 ± 1.256 mg[a]	6.33 ± 7.340 mg[a]	2.07 ± 0.490 mg
Pituitary gland	8.11 ± 1.526[a]	5.70 ± 0.361 mg[a]	2.20 ± 0.497 mg

The macroscopic phenotype in males from AROM$^+$ lines 21 and 33 was analyzed at the age of 4 months. The phenotype was consistent in all mice analyzed. The organ weights were collected from 6–12 specimens, and data show the mean ± SD.

[a] $P \leq 0.001$ *vs.* control.

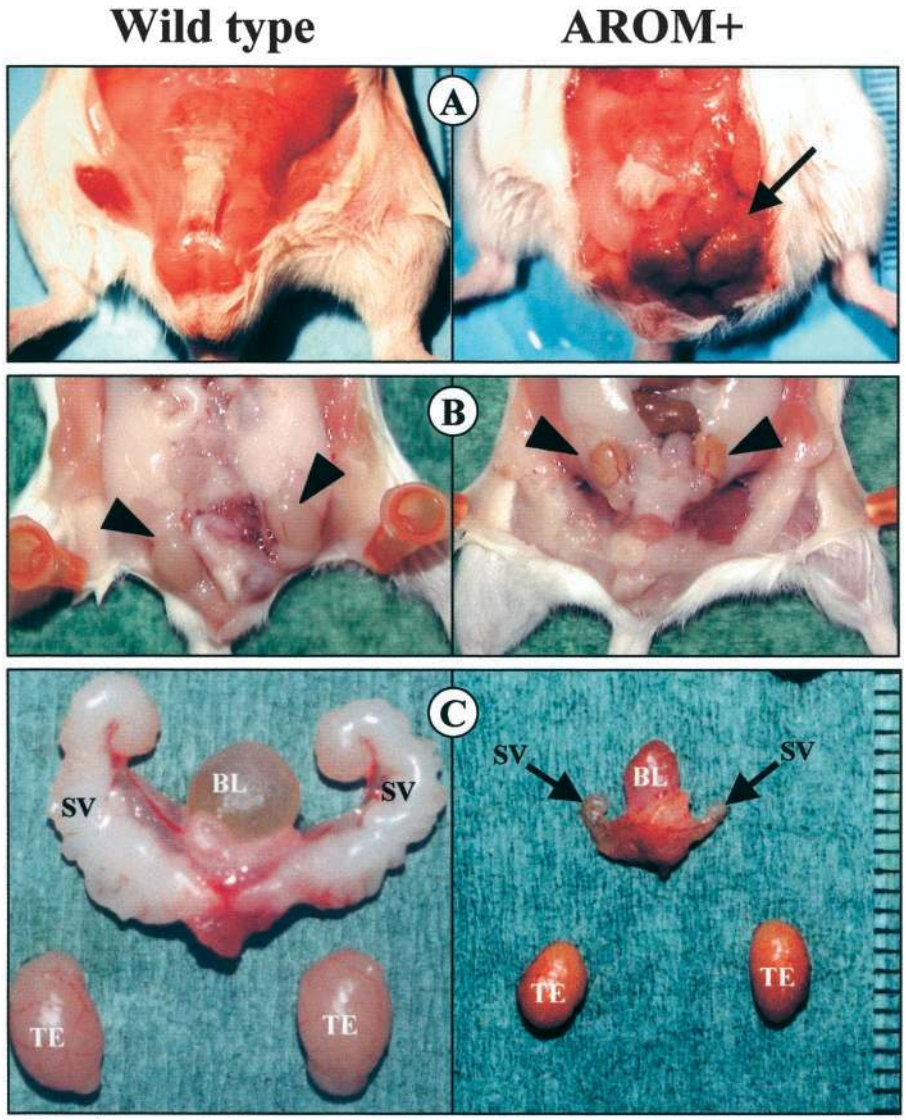

FIG. 3. A, Inguinal hernia (indicated with *arrow*) in an infertile adult AROM$^+$ male founder. B, Undescended testes in adult 4-month-old AROM$^+$ males (line 21) were found in the F_1 generation and thereafter. The testes are indicated with *arrowheads*. C, Adult AROM$^+$ males also present underdeveloped seminal vesicles (SV) and small testes (TE). BL, Urinary bladder. Age-matched WT controls are shown on the *left*.

mous epithelium was observed (Fig. 5D). In addition, pronounced expansion of the extracellular spaces in the stromal layer of ejaculatory ducts was frequently seen bi- or unilaterally (Fig. 5E).

Discussion

Male mice with ubiquitin C/P450arom transgene expression (AROM$^+$ mice) developed severe abnormalities in various parts of their reproductive organs and were infertile. The enhanced P450arom expression was shown to lead to highly elevated serum E_2 concentrations, whereas T concentrations were reciprocally decreased. Thus, the AROM$^+$ mouse model provides a novel tool to investigate the consequences of prolonged imbalance in the serum androgen to estrogen ratio and, in particular, of excessive estrogen exposure on male reproductive organs and functions. The topic is of general interest as there have been several reports on endocrine disrupters that may estrogenize males of various species, leading to disturbances in reproductive functions and behavior (28). However, it is still not known whether these abnormalities are due to the hormonal or toxic effects of these compounds, and the mouse model generated could be used to discriminate these two components.

Recently, another transgenic mouse model with P450arom overexpression was developed using mouse mammary tumor virus promoter (29). Mouse mammary tumor virus is active in male reproductive tissues as well as in the mammary gland. About half of these male mice (int-5/aromatase) were infertile and/or had enlarged testes. Histological analysis showed the mice to have Leydig cell hyperplasia and Leydig cell tumors unilaterally or bilaterally (30). However, serum E_2 levels measured in the int-5/aromatase mice were much lower than those in the AROM$^+$ mice generated in the present study (5.7 and 150 pg/ml, respectively), and it is likely that this difference is not explained solely by methodological differences. The difference in the peripheral concentrations of E_2 between the AROM$^+$ mice and int-5/aromatase mice most probably bears a biological significance as regards the development of the phenotype. In contrast to the severe structural and functional alterations in AROM$^+$ mice,

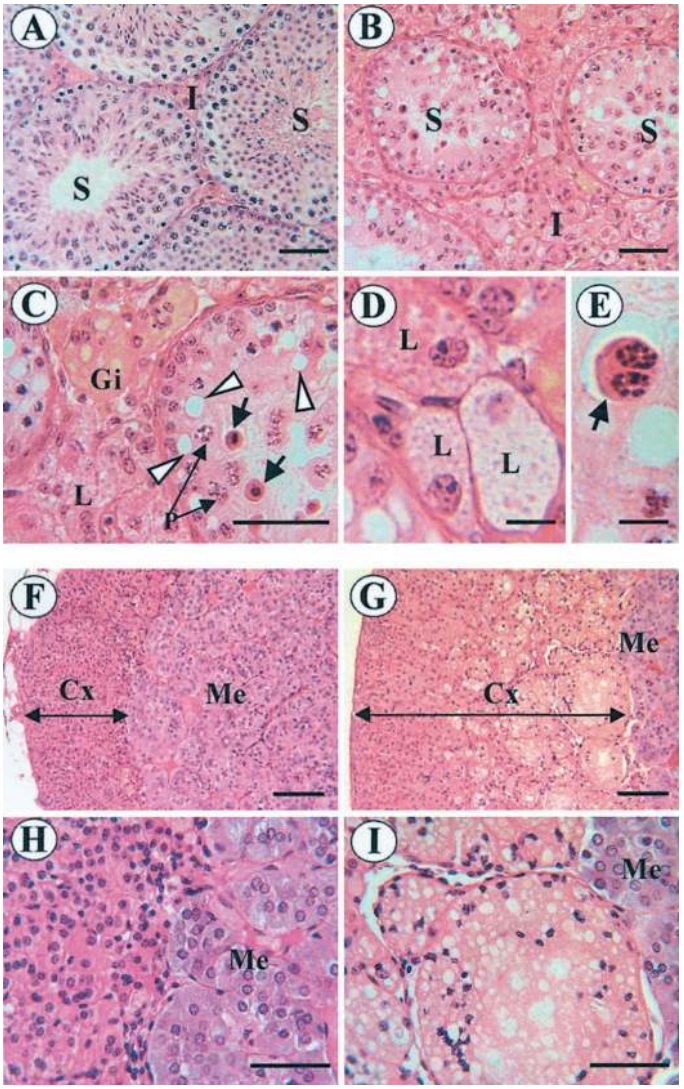

FIG. 4. Testicular and adrenal gland histology of adult WT and AROM$^+$ (line 21) male mice at the age of 4 months. A, WT mouse testis. S, Seminiferous tubule; I, interstitial cells. B and C, The volume of interstitium (I) is increased in AROM$^+$ mice and contain enlarged Leydig cells (L) and giant cells (Gi) with multiple nuclei. In addition, degenerating germ cells within the seminiferous epithelium and numerous vacuoles (*arrowheads*) of different sizes are present in AROM$^+$ testis. There are no germ cells beyond the stage of pachytene spermatocytes (P). Cells with intensively stained nuclei and eosinophilic cytoplasm (*arrows*), morphologically resembling eosinophil leukocytes, are frequently present in the seminiferous epithelium of AROM$^+$ mice. D and E, Higher magnifications showing enlarged Leydig cells (L) and eosinophil-like cells (*arrow*), respectively. F and H, WT mouse adrenal gland. G and I, Adrenal gland of AROM$^+$ mice (line 21) displaying adrenocortical hyperplasia. Low-power magnifications (F and G) through the adrenal cortex (Cx), demonstrates the increased cortical thickness in AROM$^+$ mice. The high magnification (H and I) show the pronounced expansion of the innermost cortical layer in AROM$^+$ mice, filled with large centripetal vacuole-filled structures. Me, Adrenal medulla. *Bars*, 100 μM (F and G), 50 μM (A-C, H, and I), and 10 μM (D and E).

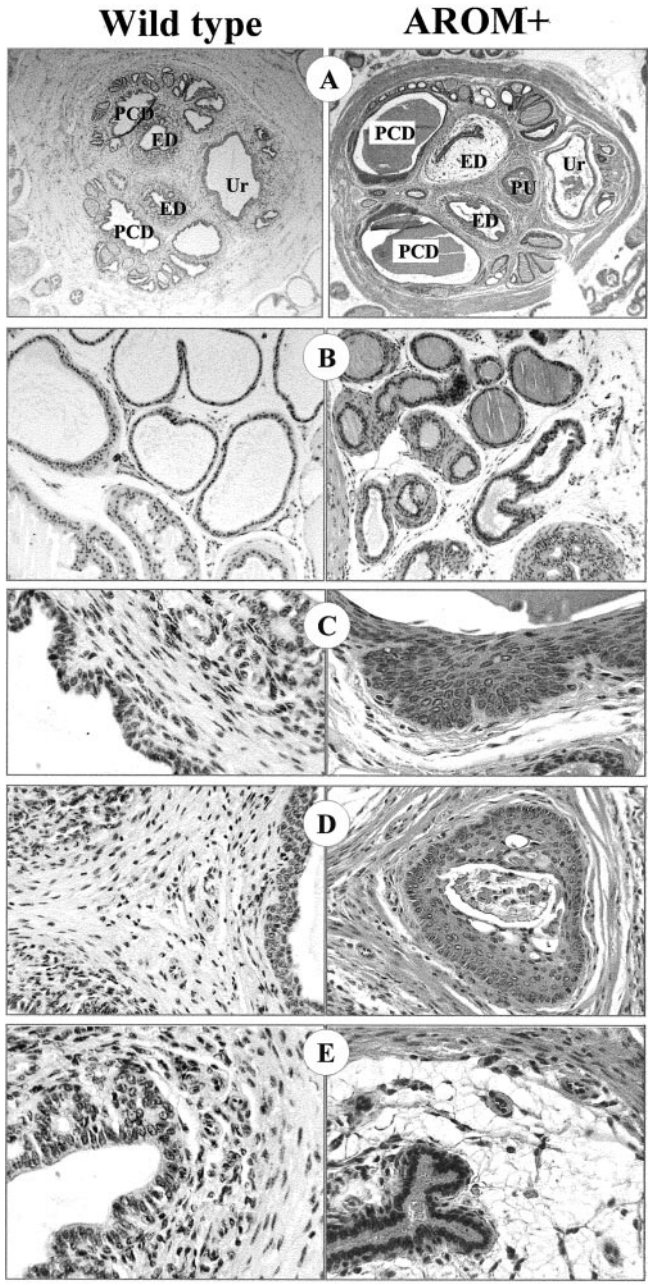

FIG. 5. Morphology of the urethroprostatic complex in a 4-month-old AROM$^+$ male mouse (*panels on the right*). The corresponding structures in an age-matched WT mouse are shown on the *left*. Transverse section through prostatic urethra (A), dorsolateral prostate (B), posterior collecting ducts (C), prostatic utricle (D), and ejaculatory ducts (E). Ur, Urethral lumen; ED, ejaculatory ducts; PCD, posterior collecting ducts; PU, prostatic utricle. In the AROM$^+$ male there is poorly developed prostate gland (B), squamous epithelial metaplasia in the collecting duct (C), a prominent prostatic utricle (indicated by PU in A, and shown also in D), and abnormal swelling/distention of the connective tissue surrounding the ejaculatory duct (E). Original magnifications, ×25 (A), ×100 (B), ×200 (C), and ×400 (D and E).

no changes of the male accessory sex glands were reported in int-5/aromatase mice. In addition, in AROM$^+$ mice PRL and corticosterone production is also markedly elevated, whereas these effects were not reported in the int-5/aromatase mice.

The AROM$^+$ males display several of the changes observed in males perinatally exposed to estrogens (19–23), such as undescended testes, testicular interstitial cell hyperplasia, hypoandrogenism, and growth inhibition of accessory sex glands. The AROM$^+$ mice were generated by using

the human ubiquitin C promoter, which is known to be activated at around embryonic day 15 in mice, and which has been shown to drive constitutive expression of transgenes in a wide range of tissues (24). This, together with the phenotypic alterations found in the present study, indicates that the estrogen to androgen ratio in AROM$^+$ mice is also high in the fetal and neonatal periods. In addition, as a sign of long-term excessive estrogen exposure in adulthood, the AROM$^+$ males showed pronounced squamous epithelial metaplasia in periurethral collecting ducts. Interestingly, no hyperplastic or dysplastic lesions were observed in the prostate glands of AROM$^+$ mice, although both perinatal estrogen treatment, as well as chronic combined estrogen-androgen treatment have been shown to induce prostatic neoplasia in rodents (31). This could be a result of the constitutive low T concentration in the AROM$^+$ males.

Disruption of spermatogenesis in AROM$^+$ mice could be a consequence of multiple factors, including cryptorchidism, abnormal Leydig cell function, hypoandrogenemia, or hyperestrogenemia. Estrogens are known to inhibit Leydig cell development, growth, and function, resulting in suppression of androgen production (32), and P450arom-deficient mice develop Leydig cell hyperplasia/hypertrophy (12). However, the present findings, together with those from int-5/aromatase mice (30), show that increased E_2 to T ratio, including excessive estrogen exposure, disrupt Leydig cell function and can cause hyperplasia, hypertrophy, and Leydig cell tumors. Consistent with these findings, both prenatal exposure to DES and chronic exposure to DES in adulthood have been shown to induce Leydig cell tumors in mice (21, 33). Numerous degenerating germ cells and no spermatids within the seminiferous tubules suggest that germ cell development was arrested at the stage of pachytene in the cryptorchid testes of AROM$^+$ mice. Interestingly, the spermatogenic arrest occurs at a stage where P450arom is typically expressed (34). However, the direct role of P450arom as a local autocrine/paracrine modulator of spermatogenesis remains to be characterized further. The spermatogenic arrest found in the AROM$^+$ mice could be explained, at least partially, by the suppressed FSH action. The morphology of the hypertrophic Leydig cells in AROM$^+$ mice was very different from that in the WT mice. The cryptorchidism alone does not lead to hyperplastic Leydig cells as shown by the relaxin-like factor knockout mice (35, 36). Hence, the structural and functional changes in the Leydig cells of AROM$^+$ males are suggested to be related with the high E_2 levels both prenatally and at adulthood. Given that the number of Leydig cells in experimentally cryptorchid testes is increased and T production suppressed (37); however, it is possible that the impaired Leydig cell function is partly associated with the cryptorchidism rather than to a direct effect of E_2 on Leydig cells.

The reduced serum FSH levels in AROM$^+$ males are further proof of the role of estrogens in suppressing FSH secretion in males (1). No significant differences in the average LH concentrations were seen between AROM$^+$ and WT male mice. However, there was less variation in serum LH levels in AROM$^+$ mice as compared with the WT males suggesting the possibility of reduced LH amplitude in AROM$^+$ male mice. A similar effect on LH amplitude has been found in men after an exogenous estrogen administration (38). The data suggest that, similar to the situation found in men (39), FSH is more sensitive than LH in regard to the suppressive effect of E_2 in AROM$^+$ males. Therefore, the AROM$^+$ mice provide a novel tool to further study the effects of estrogens on the regulation of LH and FSH secretion. Despite the normal LH concentrations, androgen levels were reduced in AROM$^+$ mice, which is in agreement with the idea of testicular failure.

AROM$^+$ males also display pronounced adrenocortical hyperplasia with enlarged cells throughout the cortex, and development of large centripetal vacuole-filled structures in the innermost cortical layer. Morphologically, this resembles the adrenal phenotype we recently observed in female bLHβ-CTP mice overexpressing LH. These mice showed high serum corticosterone concentration associated with hyperactive adrenal function due to chronic adrenal gland stimulation (40). In addition to high corticosterone and LH, bLHβ-CTP mice present elevated E_2, T, and PRL concentrations. Interestingly, in the AROM+ male mice, the adrenocortical stimulation was associated with high circulating levels of E_2 and PRL; hence, the role of these hormones in the etiology of adrenocortical hyperplasia in these mouse models remains to be explored.

The high PRL concentration found in the AROM$^+$ males is in line with previous observations showing that estrogen exposure, both at neonatal period and at adult age, may cause hyperprolactinemia in male rats (41, 42). Furthermore, together with estrogens and androgens, increased PRL action has been shown to affect prostate structure and function, and the effect is dependent on the age of the exposure. Neonatal exposure to estrogens induces hyperprolactinemia and decreases prostate weight (43), whereas a chronic (15–20 weeks) estrogen treatment of adult male rats induces persistent hyperprolactinemia, and increased prostate weight (41). The difference is likely to be due to the interaction between sex steroid and PRL as, for example, androgens are necessary for PRL induced prostatic growth (44). Also hyperprolactinemic male mice have enlarged accessory sex glands, including the prostate, due to an increased amount of secretory material and interstitial tissue (45, 46). The fact that the prostate in the AROM$^+$ mice is small and undeveloped is most likely due to the lack of proper amount of androgens at adult age and to the increased estrogen action at the neonatal period.

In conclusion, the AROM$^+$ male mice generated show complex hormonal disturbances with multiple structural and functional abnormalities in the male reproductive system. Several of the abnormalities found resemble those described in mice exposed to exogenous estrogens during perinatal life. Hence, AROM$^+$ mice represent a valuable model for analyzing the direct and indirect effects of unbalanced estrogen and androgen action, especially in the developing males.

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