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Phenotype characteristics of transgenic male mice expressing human aromatase under ubiquitin C promoter☆

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Abstract

To study the significance of the increased ratio of the estrogen/androgen concentration for the male reproductive functions, we have generated transgenic mice expressing human P450 aromatase under a promoter providing ubiquitous and permanent transgene expression (AROM+ mice). AROM+ male mice are characterized by elevated serum estradiol and prolactin (Prl) concentrations, combined with markedly reduced testosterone levels. The mice are present with a multitude of structural and functional alterations in the reproductive organs such as cryptorchidism, Leydig cell hyperplasia, disrupted spermatogenesis and infertility. Furthermore, the mice develop infravesical obstruction associated with the rhabdosphincter atrophy and rudimentary accessory sex glands. Interestingly, the mammary gland in AROM+ males undergo a ductal and alveolar development morphologically resembling terminally differentiated female mammary glands, and express several signaling proteins typical for female mammary glands. Some of the abnormalities seen in AROM+ mice are similar to those described in both mice and humans exposed to diethylstilbestrol (DES) in utero. The importance of the AROM+ model may lie in its predictability, i.e. the model suggests which abnormalities of the human reproductive functions may be associated with the increased ratio of estrogen/androgen concentrations in early life and at adult age as well.

Keywords: Transgenic mice; Estrogen; Androgen; P450 aromatase

1. Introduction

Estrogenic stimulation has been shown to be involved in several clinical manifestations in both females and males. Both human and rodent studies indicate that prenatal or early postnatal exposure to exogenous estrogens induces persistent changes in the structure and function of reproductive organs. The term developmental estrogenization syndrome has been suggested to refer to the common phenotypic features seen in diethylstilbestrol (DES) exposed offspring of both animals and humans [1]. Especially, cryptorchidism in humans has been associated with estrogen concentrations in pregnancy. A higher risk of undescended testis, smaller testis size and lower sperm count have been reported in men exposured to DES in utero than in their unexposed counter parts [2]. In addition, mothers of cryptorchid children had higher levels of free estradiol (E2) during first semester compared with mothers whose offspring had normally descended testes [3]. Moreover, recent findings on an increased level of estradiol in the human placenta of cryptorchid newborn support this concept [4,5]. On the other hand, men who are infertile with relative high estrogen concentration in serum have been successfully treated with an aromatase inhibitor [6]. Animal studies support the correlation found in humans. Estrogenization of male rodents with DES during the early developmental stages initiate structural and functional changes observable in adult animals, these include atrophic and small testes, epididymal cysts, and abnormalities in the rete testis [7–9]. Results also suggest that estrogens might have a central role in the mechanisms leading to other male reproductive tract malformations such as enlarged prostatic utricle, and diseases such as testicular [10] and prostatic tumors [11].

To further study the consequences of long-term excessive estrogen exposure on males, we have recently generated a transgenic mouse model (AROM+) expressing human P450 aromatase under the human ubiquitin C promoter (Fig. 1). P450 aromatase (P450arom) enzyme is the product

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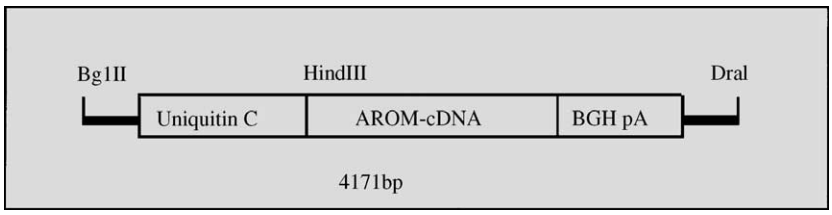

Fig. 1. Transgene construct used for overexpressing P450aromatase in transgenic mice. The transgene consists of human ubiquitin C promoter (Ubiquitin C), human P450arom cDNA (AROM-cDNA), and poly-A signal region of the bovine growth hormone (BGH pA).

of the *cyp19* gene [12]. The enzyme 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, E2 and estrone, respectively [13,14]. Together with 17β-hydoxysteroid dehydrogenase type 1 (17β HSD type 1), P450arom catalyzes the final steps in ovarian E2 biosynthesis, but these enzymes are also expressed in female and male extragonadal tissues, suggesting a role for these enzymes in the local, intracrine, estrogen production. However, extragonadal tissues lack the capacity to synthesize androgenic precursors, and estrogen production is dependent on the gonads for the supply of these precursors. In the AROM+ mice a multitude of structural and functional alterations (Table 1) were found in several male organ systems [15]. Several of the abnormalities in the urogenital organs are similar to those described in the developmental estrogenization syndrome.

Table 1
Reproductive phenotypes in AROM+ male mice

Hormonal levels
Decreased level of testosterone
Increased level of estradiol
Increased prolactin
Increased corticosterone
Urogenital tract
Infertility at all ages
Cryptorchidism
Reduced testis size
Leydig cell hyperplasia and hypertrophy
Abnormal seminiferous tubules with spermatonic arrest
Reduced size or rudimentary prostate
Rudimentary seminal vesicles
Mammary gland
Ductal elongation, branching and differentiation
Expression of β-casein
Expression of estrogen receptor-α and -β
Expression of phosphorylated Stat5
Adrenals
Enlarged adrenal gland size
Adrenal cortical hyperplasia
Pituitary
Enlarged pituitary size
Increased number of lactotrophs

2. AROM+ mice

2.1. Generation of AROM+ mice, and their reproductive characteristics

The AROM+ mice were generated in FVB/N genetic background [15]. Three of the founders (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 phenotypic characteristics of the two AROM+ mouse lines generated were identical, and hence, most of the currently available data arise from using the mouse line 21. The ubiquitin C promoter used in the generation of the AROM+ model typically give low level of expression in multiple tissues, and is prenatally activated [16,17]. Accordingly, our RT-PCR analysis demonstrated low level of transgene expression both in gonadal and extra-gonadal tissues of the AROM+ male mice [15]. 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. In contrast to males, the AROM+ females were not found to have obvious defects in their reproductive functions. They go through pregnancy, deliver pups, and nurse the offspring normally.

2.2. Hormonal levels

In our first set of experiments, the hormone levels of AROM+ male mice were analyzed at the age of 4 months [15]. Serum E2 concentrations were elevated while T concentrations were markedly reduced in serum. Serum progesterone concentrations in the AROM+ male mice were also slightly elevated, but the difference between AROM+ mice and WT mice was not statistically significant [15]. Serum FSH concentrations were found to be reduced at the age of 4 months [15], supporting the hypothesis that estrogens suppress FSH secretion in males [18]. No significant differences in the average LH concentrations were seen between AROM+ and WT male mice. However, the tendency towards a lower LH concentration was frequently seen in AROM+ mice. FSH is more sensitive than LH as regards the suppressive effect of E2 also in men [19]. The AROM+ mice could, thus, provide an interesting tool for further studies

of the role of estrogens in the regulation of LH and FSH secretion.

As compared with gonadotropins, a more marked effect was seen in serum prolactin (Prl) concentrations, which were increased over 40-fold at the age of 4 months. The number and density of Prl-producing cells were strikingly increased in AROM+ males, while the positive cells were distributed throughout the anterior pituitary [15]. The high Prl concentrations found in the AROM+ males are in line with previous observations showing that increased estrogen exposure, both during the neonatal period and at adult age, may cause hyperprolactinemia in male rats [20,21]. Interestingly, female mice overexpressing hCGβ subunit under the ubiquitin C promoter show elevated E2 levels at the age of 1–2 months, and also develop lactotroph adenomas [17], indicating a connection between increased estrogen production and the development of lactotroph adenomas in both sexes.

2.3. Testis phenotype

The testes in the newborn AROM+ mice are histologically indistinguishable from the WT testes (Fig. 2). However, at adult age all the AROM+ males were cryptorchid, with the testes located in the abdominal cavity at the inlet of the inguinal canal. Histological evaluation suggested that germ cell development was arrested at the stage of pachytene in the AROM+ mice at the age of 4 months [15]. At the age of 15 months, most of the seminiferous tubules lacked the developing germ cells (Fig. 2C and E). Furthermore, cells with intensively stained bilobulated nuclei and eosinophilic cytoplasm, morphologically resembling eosinophilic leukocytes, were present in seminiferous tubules at the age of 4 months.

The testicular interstitium was enlarged and filled with two populations of cells, namely hypertrophic Leydig cells and large multinucleated cells [15]. The Leydig cell origin of the hyperplasia was supported by immunostaining the testicular sections with an antibody against 3β-hydroxysteroid dehydrogenase type I (Fig. 3) at the age of 4 months. The 3β-hydroxysteroid dehydrogenase positive cells displayed a swollen cytoplasm, and were larger in size than those of the WT males. The phenotypes present in the mice with increased E2/T ratio suggest that excessive estrogen exposure, directly or indirectly, disrupt Leydig cell function and can cause hyperplasia, hypertrophy and Leydig cell adenomas. In addition, estrogens are known to inhibit Leydig cell development and function, resulting in suppression of androgen production [15,22,23]. Apparently, a delicate balance between E2 and T is essential, as also the P450arom-deficient ArKO mice develop Leydig cell hyperplasia and hypertrophy [24]. Consistent with the studies on gene modified mouse models, both prenatal exposure to DES and chronic exposure to DES at adulthood have been shown to induce Leydig cell tumors in mice [25,26]. It is likely that cryptorchidism alone does not lead to hyperplastic Leydig cells, as similar phenotype was not seen in

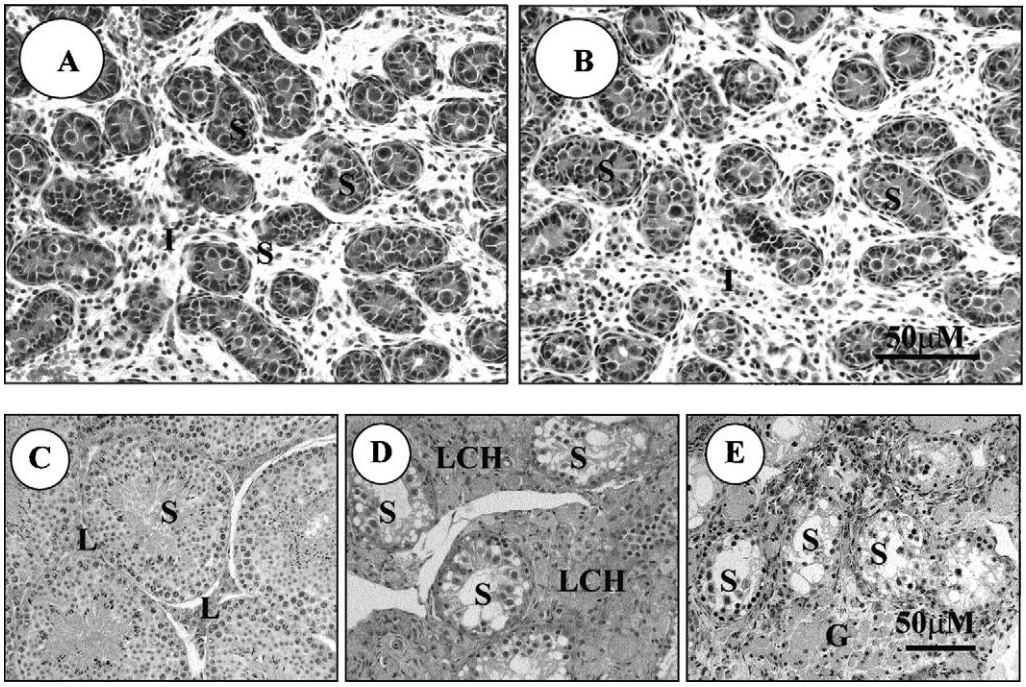

Fig. 2. Testis histology in wild type (WT) mice and in AROM+ mice. At the late fetal life (E17.5), no obvious differences could be detected in the testicular histology between the WT (A) and AROM+ mice (B). At the age of 4 months, Leydig cell hyperplasia and disrupted spermatogenesis is detected in AROM+ mice (D), as compared with the WT mouse testis (C). (E) AROM+ mice testis at 15 months of age shows present of giant cells (G), and no germ cells were present in the seminiferous tubules (S). Leydig cell (L).

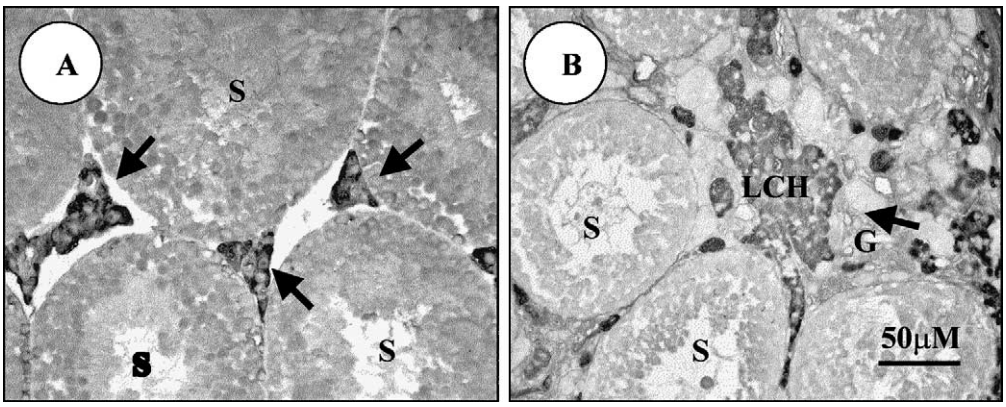

Fig. 3. Immunhistochemical staining for 3β HSD type I in the testis. (A) Wild type mouse, and (B) AROM+ mouse at the age of 4 months. The staining shows a markedly increased number of the Leydig cell in AROM+ mice. S, seminiferous tubule. LCH, Leydig cell hyperplasia. G, giant cell.

cryptorchid Insl3 knockout mice lacking the gubernaculum development [27,28]. Hence, the structural and functional changes in the Leydig cells of AROM+ males are suggested to be related with the increased E2 levels both prenatally and at adulthood. However, as the number of Leydig cells in experimentally cryptorchid testes is typically increased and T production suppressed [29], it is possible that the impaired Leydig cell function is partly associated with the E2-induced cryptorchidism rather than to a direct effect of E2 on Leydig cells. P450arom is endogenously expressed in the testis at low levels [30], but the role of P450arom as a local autocrine/paracrine modulator of spermatogenesis remains to be characterized further. Disruption of spermatogenesis in AROM+ mice could well be a consequence of multiple factors, including cryptorchidism, abnormal Leydig cell function, hypoandrogenemia or hyperestrogenemia, and also at least partially caused by suppressed FSH action.

2.4. Other male genital organs

As expected, the weights of the epididymides, seminal vesicles, and prostatic lobes were significantly reduced in AROM+ mice. Histological examination further revealed undifferentiated stratified epithelium and uncanalized bud-like formations surrounded by dense fibromuscular stroma [15]. This is well in line with previous studies showing that perinatal exposure to estrogens results in permanent suppression of prostate growth in rodents ([31], for review). The low androgen level in adult AROM+ is, of course, likely to contribute to the small size of the androgen dependent accessory sex glands. Furthermore, in the prostatic collecting ducts, squamous epithelial metaplasia was present in all analyzed AROM+ males, although the extent varied from animal to animal. In some AROM+ males a prominent prostatic utricle with keratinized stratified squamous epithelium was observed. In addition, pronounced expansion of the lamina propria of the vas deferens was frequently seen bi- or unilaterally [15]. All of these findings are consistent with earlier observations on the effects of long-term exposure to high estrogen levels.

Somewhat surprisingly, AROM+ mice do not develop prostatic tumors or hyperplasia, even though they have permanently elevated levels of estrogens and Prl. It is well established that perinatal exposure to estrogens, or long-term exposure to estrogens and androgens in adulthood, results in the development of prostatic neoplasia in the rodents ([32], for review). Furthermore, hyperprolactinemia causes pronounced prostatic enlargement in mice [33] and Prl induces epithelial hyperplasia in rat prostate [34]. At present, there is no clear explanation for the lack of prostatic neoplasia in AROM+ mice. It is likely that insufficient androgen action (AROM+ mice are hypoandrogenemic) is associated with the prostate phenotype, but further studies are needed to clarify if androgen replacement can induce normal prostatic growth or development of prostatic tumors in AROM+ mice.

2.5. Uretral dysfunction in AROM+ mice

Compared with WT mice, AROM+ mice showed higher maximal bladder pressure and decreased maximal urinary flow rate (Fig. 4), consistent with the presence of the infravesical obstruction [35]. The infravesical obstruction was associated with the rhabdosphincter atrophy and with the reduced size of the prostatic lobes [35]. These changes resemble closely those observed in neonatally estrogenized male rats [36,37], which develop non-traumatic infravesical obstruction. The findings in AROM+ model suggest that elevated endogenous estrogens (or androgen deficiency) may cause functional rhabdosphincter disorder. The treatment of adult AROM+ mice with aromatase inhibitor (finrozole, Hormos Medical Corp., Finland) for 6 weeks significantly increased the maximal flow rate in AROM+ mice, while the bladder pressure slightly decreased. Furthermore, the relative thickness of the proximal rhabdosphincter was restored after the finrozole treatment in AROM+ mice [38]. This indicates that developmentally induced urodynamic

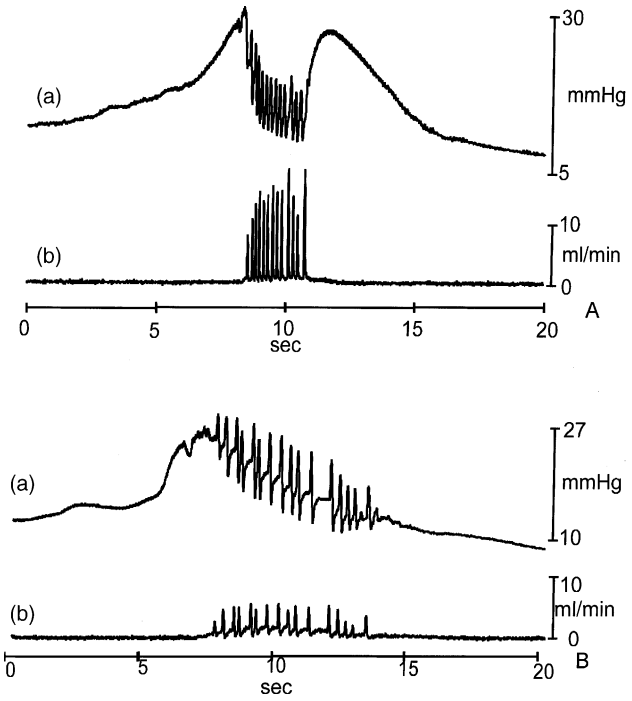

Fig. 4. Simultaneous recording of the bladder pressure (a) and flow rate (b) during typical micturination contraction of an adult wild-type male mice (A) and AROM+ males (B).

alterations in AROM+ male mice are reversible in the adulthood. DES exposed men have been reported to experience problems in passing urine, but no urodynamic studies have been conducted to elucidate the pathogenesis of the possible voiding problems [39]. Furthermore, the significance of the decrease in T and the possible increase in E2 concentrations with age is open for the development of obstructive voiding. It also remains to be demonstrated if aging men with bladder outlet obstruction would benefit from aromatase inhibitor treatment [40].

2.6. Adrenal phenotype

In addition to the disrupted urogenital organs, the structure and function of adrenals was also affected in AROM+ mice. The weights of the adrenals were significantly greater than those of control mice at the age of 4 months, and an increased corticosterone production was detected [15]. Furthermore, histological examination showed adrenocortical hyperplasia, with markedly expanded innermost cortical layer. Morphologically and functionally, these alterations resemble the adrenal phenotype recently observed in female bLHβ-CTP mice overexpressing a LH analog. Similar to male AROM+ mice, female bLHβ-CTP mice had high serum corticosterone concentrations associated with hyperplastic adrenal cortex [41]. Interestingly, also in these mice the adrenal cortex hyperplasia is associated with elevated serum E2, T and Prl concentrations. Hence, we are currently studying the role high circulating levels of E2 and Prl in the etiology of adrenocortical hyperplasia in these mouse models.

2.7. Mammary gland phenotype

Interestingly, the AROM+ male mice develop gynecomastia [42], and their mammary glands undergo ductal and alveolar development morphologically resembling those of females (Fig. 4). Mammary gland ducts were seen prepubertally as early as day 20 after birth, and at 40 days of age ducts were well organized, and the ductal tree resembled that of a normal age-matched virgin female mice. At the age of 4 months, the ducts had completed their elongation and the ductal tree filled the mammary fat pad, similarly to that in age-matched virgin WT female mice. Histological sections indicated that ducts had a single layer of epithelial cells surrounded by stromal cells. At the age of 6 months, increased tertiary side-branching and lobulo-alveolar development could be detected, and the glands structurally resembled mammary glands of WT female mice on the second week of pregnancy. At the age of 9 months, the AROM+ male mammary glands had differentiated further and resembled terminally differentiated female glands [42] typically present in late pregnancy (day 16.5 of gestation, Fig. 5).

As analyzed at the age of 4 months, stainable material was frequently seen in the lumen of the ducts. The data, furthermore, revealed that β-casein mRNA was expressed in the mammary gland of AROM+ males. Immunohistochemical analysis at the age of 4 months showed the positive staining for estrogen receptor-α (ERα) in the epithelial cells of both ducts and alveoli. A similar cellular distribution was observed for ERβ, and progesterone receptor (PR), which were also frequently detected in the alveolar and ductal epithelial cells. We have further shown the phosphorylation of Stat5 proteins in AROM+ mammary glands at the age of 4 months, indicating that these Prl signal transducers were activated upon stimulation with Prl. Intense positive staining was found in the nuclei of epithelial cells, especially in the alveoli, similar to that found in the mammary glands of pregnant WT female mice [42]. This suggests that, like in females, Stat 5 proteins are involved in the establishment of the differentiated mammary gland in the AROM+ males with gynecomastia like phenotype [43,44]. Estrogens are apparently not required for normal prenatal mammary gland development. In contrast, T produced by the developing gonads of the male fetus is known to cause a condensation of mesenchyme around the heel of the gland, and hence, the mammary gland in the male starts to regress on gestational d13 in mice [45]. Excessive estrogen production, starting prenatally in AROM+ males, could, thus, interfere with the normal T action required for regression. In the absence of T-induced regression, the mammary gland is known to maintain its full competence for the development in male mice.

Our results are consistent with the idea that estrogens play a major role in promoting elongation of the mammary ducts

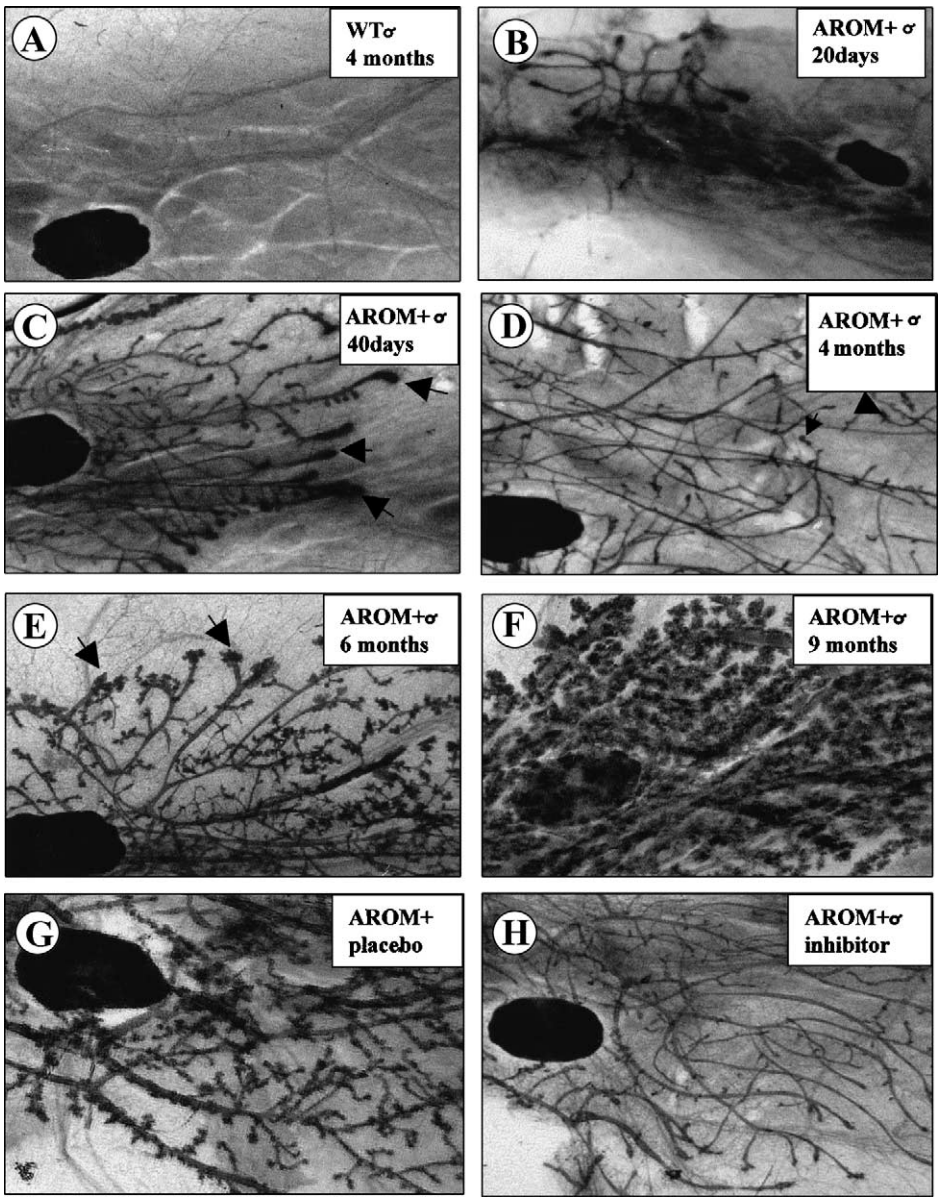

Fig. 5. Mammary gland structure in AROM+ mice: (A) The mammary fat pad of the WT male mice is devoid of ductal structures. (B) At the age of 20 days, few rudimentary ducts are present proximal to the lymph node. (C) Prominent terminal end buds (TEBs) are visible at the age of 40 days (*arrow*). (D) At the age of 4 months, the ducts have reached the border of the fat pad. At this stage the TEBs have disappeared. (F) At the age of 9 months, extensive lobuloalveolar development occurs that resembles the structures present in female mammary gland at late gestation. At this stage, the mammary fat pad is completely filled with the secretory lobuloalveolar structures. Arrows indicate the presence of alveolar structures.

[46]. Together with progesterone, estrogens are also required for the development of tertiary ducts, and for the maintenance of ducts and lobulo-alveolar structures in females [47]. Interestingly, the mammary phenotype of the AROM+ male mice was partially reversible. A 6 week treatment with finrozole, a novel and specific inhibitor of aromatization [48] suppressed the formation of the alveolar structures and the ducts lacked the majority of the tertiary side branches [42]. This further substantiates the primarily role of estrogens in the development and maintenance of the mammary gland in AROM+ male mice.

E2 could also have a permissive role for the action of progesterone [49,50] in the AROM+ mammary gland. In mammary glands of the PR-deficient mice, the ductal structure has less extensive side branching and acinar development, and lacks interductal lobuloacinar structures [51,52]. Progesterone concentrations of the AROM+ male mice were not significantly elevated when compared with the WT male mice, and were lower than in females, but obviously sufficient for an efficient activation of the PR mediated signaling systems in the AROM+ mammary gland [42]. The presence of certain markers, such as the expression of Npt2b

and β-casein suggested a differentiation towards the alveolar epithelium. The hormonal causes of gynecomastia are still poorly understood. Especially, the possible direct and antagonistic actions of androgens on estrogen action or the androgen-independent action of estrogens remains to be explored. AROM+ male mice provide an animal model with a condition resembling gynecomastia which could be used for better understanding the cellular and molecular mechanisms involved in the development of gynecomastia, as well as for implementation of preclinical studies on treatments affecting the androgen-estrogen balance, such as aromatase inhibitors, antiestrogens or non-aromatizable androgens [53].

Even though the signaling pathways for estrogens, progestins and Prl are present in the AROM+ male mammary gland, no sign of carcinogenesis was detected during the 9-month long follow-up period. This is interesting as the female transgenic mice expressing Prl do develop mammary tumors. For some reason the proliferative pressure of the altered hormonal environment in AROM+ males was not sufficient for the initiation of the immortalization of the epithelial cells in AROM+ male mammary gland.

3. Conclusions

The AROM+ male mice show complex hormonal disturbances with structural and functional abnormalities in multiple organ systems. Several of the abnormalities resemble those described in mice exposed to exogenous estrogens during perinatal life. AROM+ male mice provide a novel tool for investigating the significance of increased ratio of the estrogen/androgen concentrations in the males, and especially to study the mechanisms of Leydig cell hyperplasia, infertility, lower urinary tract dysfunction or gynecomastia. Crossing of AROM+ mice with knockout mice deficient in the signaling systems for various hormones (e.g. ER-knockout mice) would be essential to future elucidate the mechanisms involved in the estrogen related dysfunctions identified in AROM+ males.

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