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Androgen Resistance in Female Mice Increases Susceptibility to DMBA-Induced Mammary Tumors

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Abstract Hormones, notably estrogens, are pivotal in the origins of breast cancer but androgenic effects, while supported by persistence of AR expression in breast cancers, remain controversial. This study determined the role of the androgen actions via androgen receptor (AR) in experimental mammary cancer. Androgen-resistant female and male mice (ARKO) were generated using Cre/loxP technique and featured a global AR inactivation. The effect of AR inactivation and influence of genetic background on 7,12-dimethylbenz[a]anthracene (DMBA)-induced tumorigenesis was confirmed using two separate ARKO models with different genetic backgrounds. The onset of palpable mammary tumors was significantly faster in ARKO females (median time 22 vs 34 weeks, respectively; (p=0.0024; multivariate Cox regression) compared to WT and independent of the mouse genetic background. The cumulative incidence at 9 months was 81±10% [mean±SE] for ARKO compared to 50±13% in WT females. The increased DMBA susceptibility of ARKO females was associated with a higher epithelial proliferation index but not with major structural or receptor (estrogen or progesterone) expression differences between the virgin WT or ARKO female mammary glands. AR inactivation allowed substantial ductal extension in ARKO males while WT males displayed only rudimentary epithelial branches or complete regression of epithelial structures. Yet, DMBA did not induce epithelial mammary tumors in WT or ARKO males, demonstrating that AR inactivation alone is insufficient to promote mammary tumors. These results demonstrate that AR inactivation accelerates mammary carcinogenesis in female mice exposed to the chemical carcinogen DMBA regardless of mouse genetic background but require prior exposure to endogenous ovarian hormones.

Keywords Breast cancer · Mammary gland · Androgen receptor · Carcinogenesis · 7,12-Dimethylbenz[a]anthracene · Cre/loxP · Mouse model

Abbreviations

DMBA	7,12-dimethylbenz(a)anthracine
ARKO	androgen receptor knockout
WT	wild-type
AR	androgen receptor
PR	progesterone receptor
ER	estrogen receptor
PCNA	proliferating cell nuclear antigen
tfm	testicular feminized

Introduction

The specific mechanisms in mammary gland tumorigenesis remain speculative with the strongest clues arising from endogenous reproductive hormone levels as epidemiological risk factors [20, 79]. While estrogens have a widely understood involvement [72, 92], the role of androgens remains controversial [8, 32, 49]. In women, circulating androgens are generated directly by secretion from the ovary

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and adrenal glands during the reproductive years and mainly by the adrenal glands after menopause [22]. In addition, androgens are produced indirectly by extra-glandular conversion of precursor steroids with the principal androgen, testosterone, being the obligate steroidal precursor of estradiol (E2), the main estrogen [33, 78]. Yet all androgens exert their distinctive biological effects via the singular common pathway of the androgen receptor (AR) [10, 41].

AR is prominently expressed in developing and mature mammary cells of women and mice [17, 31, 64]. During the embryonic development of mammary gland, the AR is expressed in mesenchymal cells and responds to androgens causing regression of the mammary development in males [61]. In the postnatal mammary gland AR is present mainly in epithelial cells [48, 63] with the expression increasing during maturation [63]. AR is also reported to be present in 70–90% of breast cancers, comparable with the observed high prevalence of estrogen receptor α (ERα; 70–80%) and progesterone receptor (PR; 50–70%) positivity [27, 43, 45, 59]. However, up to 25% of women with metastatic breast cancer is positive for AR, but not ERα or PR [19, 44]. The persistence of AR expression and/or increased endogenous androgen exposure suggests a role for AR in the pathogenesis of breast cancer [66].

Androgen action in the breast is complex. An androgen can act either directly by activating AR within the mammary gland, or indirectly by serving as a substrate for estrogen synthesis with subsequent estrogen action on mammary glands, a classical estrogen target tissue. Many androgens including testosterone can be aromatized into corresponding estrogens, with potential ER-mediated actions [90]. This makes it difficult to define the precise molecular mechanism(s) involved solely by pharmacological means. The complexity of androgen effects and lack of decisive models has made it difficult to unambiguously define androgen actions in specific stages of breast cancer progression. While knockout models have been used to determine the role of PR and ERs in mammary biology and pathology [5, 12], global ARKO female mice homozygous for an inactivated AR cannot be produced by natural mating. This is due to sterility of hemizygous males with an inactive AR that exhibit classical complete androgen insensitivity phenotype, formerly known as testicular feminization (tfm). However, this limitation has been overcome using Cre/loxP recombination technique [90], revealing the role of androgens in female physiology [36, 74, 88].

Therefore, to provide clear evidence on the in vivo role(s) of AR-mediated androgen actions in mammary tumorigenesis, we combined our androgen-resistant AR knockout (ARKO) mouse model with the standard chemical carcinogenesis model using 7,12-dimethylbenz(a)anthracine (DMBA). Our systemic ARKO females are produced using the Cre/loxP system to create a minimally truncated AR protein lacking the exon 3 which specifies the second zinc finger so that the mutant AR is unable to bind to DNA [88]. This allowed generation of globally androgen-resistant females, which cannot be bred by natural mating. These ARKO females lacking 2nd Zn finger of AR are subfertile with a predominant defect in ovulation rates like all other ARKO female models published so far [36, 74, 88], but lack the accelerated ovarian failure [88] reported in another ARKO female model [74]. Despite the differences in ARKO female phenotype, all ARKO male mice display complete androgen insensitivity phenotype demonstrating that the AR is inactive and therefore the differences are assumed to be due to secondary consequences of either lacking the AR protein or having presence of transcriptionally inactive AR protein [90]. DMBA is a widely used carcinogen model [50, 71] and generates AR positive [39] and androgen responsive [14, 16] mammary tumors in rodents.

Methods

Generation of ARKO Mice $ARKO^{Cmv}$ females and males homozygous for global AR inactivation were generated using Cre/loxP system as previously described [88]. In addition, the effect of AR inactivation and influence of genetic background on DMBA-induced tumorigenesis was confirmed using another ARKO model generated by crossing mice bearing loxP flanked AR exon 3 (ARf^{Ex3}; C57BL/6J background; [58]) with transgenic Sox2-Cre (FVB/N; [30]) mice as a universal deletor ($ARKO^{Sox}$). Wild-type (WT) females and males from the respective colonies WT^{Cmv} and WT^{Sox} were used as controls. The global AR inactivation in $ARKO^{Sox}$ mice was confirmed by development of tfm phenotype in $ARKO^{Sox}$ males. Comparable to $ARKO^{Cmv}$ males, $ARKO^{Sox}$ males had female external phenotype, small (~10% of WT) abdominal testes and spermatogenesis arrested at the pachytene stage (Supplemental Fig. 1).

Ethics All procedures were approved by the Sydney South West Area Health Service Animal Welfare Committee within National Health Medical Research Council guidelines for animal experimentation.

Experimental design WT^{Cmv} and WT^{Sox} as well as $ARKO^{Cmv}$ and $ARKO^{Sox}$ females and males were exposed to six weekly doses of 1 mg DMBA (in 100 µl sesame oil vehicle) delivered by gavage from 8 weeks of age (Supplemental Fig. 2A). The DMBA delivery was performed without regard to estrus stage. Mice were examined weekly for palpable mammary tumors and sacrificed at 9 months after DMBA treatment, unless the tumors reached ~1 cm in diameter or mice were moribund at an earlier age. Untreated controls received sesame oil vehicle only. DMBA-induced

tumorigenesis was determined in each ARKO line and the respective WTs separately as well as combined as ARKO ($ARKO^{Cmv}$ and $ARKO^{Sox}$) and WT (WT^{Cmv} and WT^{Sox}). Number of palpable mammary tumors was recorded at necropsy.

For analysis of intact mammary glands, the 4th inguinal mammary glands were collected from virgin 8 weeks old WT^{Cmv} and $ARKO^{Cmv}$ female mice at the estrus stage of estrous cycle. Estrous cycling was determined in sexually mature females by light microscope analysis of vaginal epithelial cell smears [54]. In addition, the 4th inguinal mammary glands were collected from 8 weeks old WT^{Cmv} and $ARKO^{Cmv}$ males.

To confirm the presence of inactivated Ar in the mammary glands, the expression of mutated, exon 3 deleted Ar, or intact Ar in the mammary glands was determined by RT-PCR as previously described [76]. Exclusively exon 3 deleted Ar (Ex3ΔAR) product was confirmed in 8 weeks old $ARKO^{Cmv}$ mammary glands while larger Ar product with intact exon 3 (wt AR) was detected in WT^{Cmv} mammary glands (Supplemental Fig. 2B, C). Similar results were obtained for $ARKO^{Sox}$ and WT^{Sox} mammary glands (data not shown).

Tissue Preparation, Histology, and Whole Mount Mammary glands with or without tumors were removed and either snap frozen in liquid nitrogen for mRNA (stored in −80°C) or fixed in 4% paraformaldehyde at 4°C overnight. Paraffin-embedded tissues were sectioned to 5 μm and stained with hematoxylin and eosin (H&E). Histopathological analysis was performed from H&E stained sections by an experienced pathologist (GW). For whole mount analysis, the inguinal mammary glands were dissected free from abdominal wall and skin, and spread on a glass slide. Tissues were fixed overnight in Carnoy's fixative, rehydrated, and stained in carmine alum stain. Stained glands were dehydrated, cleared in xylene, and cover slipped for microscopical analysis [55]. Ovaries were weighed at the time of collection.

Ductal Extension and Branch Points The extent of ductal expansion was analyzed based on the methods previously described [47]. Briefly, ductal distance was measured perpendicularly from the center of lymph node to the furthest end of the ductal tree. The number of epithelial branch points was quantified within the 8 mm^2. The tangent of the square was drawn perpendicularly from the distal end of the lymph node towards the furthest end of the ducts.

Immunohistochemistry For immunolocalization of ERα, PR, cytokeratin 8 (CK8), and smooth muscle α-actin (SMA), the primary antibodies used were rabbit anti-ERα (sc-542, 1:200; Santa Cruz), rabbit anti-PR (sc-538, 1:200; Santa Cruz), rat anti-CK8 (Developmental Studies Hybridoma Bank, Iowa, USA), and mouse anti-SMA (Sigma, Sydney, Australia) visualized using anti-rabbit secondary antibodies as previously described [77]. The rat anti-CK8 was detected using goat anti-rat secondary antibody accompanied with DAB detection as previously described [77]. All antibodies required microwave based antigen retrieval with 10 mM citric acid (pH 6; 15 min).

Stereological Quantification CASTGRID V1.10 (Olympus Corp. Albertslund, Denmark) software was used for unbiased stereological quantification of epithelial cells positive for ERα or PR as previously described for prostate epithelial proliferation [76]. Sampling was conducted at uniform random intervals along x- and y-axes of the cross section of mammary gland using software generated counting frames and a point grid. At least 300 epithelial cells were counted and marked as positive or negative.

mRNA Expression mRNA extraction, cDNA conversion and real-time RT-PCR were performed as previously described [77]. The following primers were used for analysis of ERα and PR expression: ERα-forward (5′-CCAGACTGC AAGCCCAAATGT-3′) and ERα-reverse (5′- CACCCA TTTCATTTCGGCCTTC-3′) and PR-forward (5′-CAGATTC AGAAGCCAGCCAGAG-3′) and PR-reverse (5′-CCACAG GTAAGCACGCCATAG-3′). The mRNA expression of CyclinE1, CyclinD1, and $p21^{Waf1}$ was analyzed using SABioscience's RT2 Real-Time PCR kits (Jomar Bioscience Pty Ltd, SA Biosciences, Australia) as previously described [76, 77].

Serum Hormone Assays Serum levels of testosterone, E2, DHT, and its two principal metabolites 5α-androstane-3α,17β-diol (3αdiol) and 5α-androstane-3β,17β-diol (3βdiol) were analyzed in female mice treated with DMBA (carrying mammary tumors at the time of collection) and vehicle (sesame oil)-treated females. Steroids were measured in extracts of 100 μl mouse serum by liquid chromatography tandem mass spectrometry (LC-MS/MS) [29] as adapted for mouse serum [53]. Serum was extracted with 3:2 (volume/volume) of hexane/ethyl acetate fortified with testosterone-1,2,3-d_3 (d_3-T), dihydrotestosterone-16,16,17-d_3 (d_3-DHT), and estradiol-2,4,16,16-d4 (d4-E2) as internal standards. The organic layer, separated by freezing the aqueous layer, was dried and reconstituted in 1.2 ml of 20% methanol in PBS with 1.0 ml injected onto the C8 column for analysis. The level of quantification (LOQ) for testosterone, DHT, and E2 were 20, 100, and 5 pg/ml, respectively.

Mouse serum LH (mLH) was analyzed using an immunofluorometric assay as previously described [86] but modified to use mLH antibodies [40]. The capture antibody used is the anti-LH antibody (5303 SPRN-1, Medix Biochemica, Turku, Finland) and the detection antibody is the anti-LH

antibody (MAb 518B7, supplied by Dr J Roser, Department of Animal Science, UC Davis, [81]) directly labeled with a Europium chelate using the DELFIA Eu-labeling kit (Perkin Elmer, City, Country) as per suppliers methodology. For the mLH assay, the detection limit was 0.02 ng/ml, the quantification limit 0.05 ng/ml, and the within-assay QC was 6.8% at low (0.25 ng/ml), 4.7% at mid (0.49 ng/ml), and 7.4% at high (1.18 ng/ml) range. Mouse serum FSH was determined using a specific immunofluorometric assay as described and validated previously [40]. All immunoassays were performed in a single batch.

Data Analysis and Statistics Data are shown as mean±SE or mean and 95% confidence intervals unless otherwise stated. In case of non-detectable serum T levels, for analysis those samples were given a value half of the detection limit (10 pg/ml). The statistical analyses were performed using Kaplan–Mayer survival analysis or analysis of variance (ANOVA; SPSS Inc, Chicago, IL). p values less than 0.05 were considered statistically significant.

Results

Independent of genetic background, AR inactivation accelerates DMBA-induced mammary carcinogenesis in female mice. Treatment with DMBA induced mammary pathology in female mice detected by early mammary ductal and alveolar gross lesions (whole mount; Fig. 1a) and development of palpable tumors as early as 2 weeks following the last DMBA dose (Fig. 1b, c). The emergence of DMBA-induced palpable mammary tumors did not significantly differ between WT^{Cmv} and WT^{Sox} females (p=0.756) or between $ARKO^{Cmv}$ and $ARKO^{Sox}$ females (p=0.533) (Table 1; Supplemental Fig. 3) and therefore the mammary tumorigenesis was determined as combined data for ARKO ($ARKO^{Cmv}$ and $ARKO^{Sox}$) and WT (WT^{Cmv} and WT^{Sox}). The cumulative incidence at 9 months was 81±10% [mean±SE] in ARKO females compared with 50±13% in WT females (Fig. 1c). The latency to palpable mammary tumors was significantly (p=0.024; multivariate Cox regression) reduced in ARKO females with median time for 50% of mice to develop mammary tumors was 34 weeks for WT (n=39) and 22 weeks for ARKO (n=36; Fig. 1c; Table 1). The number of palpable mammary tumors (per tumor positive mouse) was not significantly different between the WT (1 [1, 2] (median [range])) and the ARKO (1 [1–3]) females (Fig 1d). No apparent variation was observed in tumor growth between WT and ARKO, but without detailed analysis of tumor growth rate, possible differences cannot be excluded. AR inactivation alone did not predispose to mammary cancers, as ARKO or WT females treated with vehicle sesame oil without DMBA did not develop any tumors (data not shown).

Contrary to females, neither WT nor ARKO males developed palpable mammary tumors following DMBA treatment (n=29 for WT and 27 for ARKO males, data not shown). However, enlarged mammary gland-associated

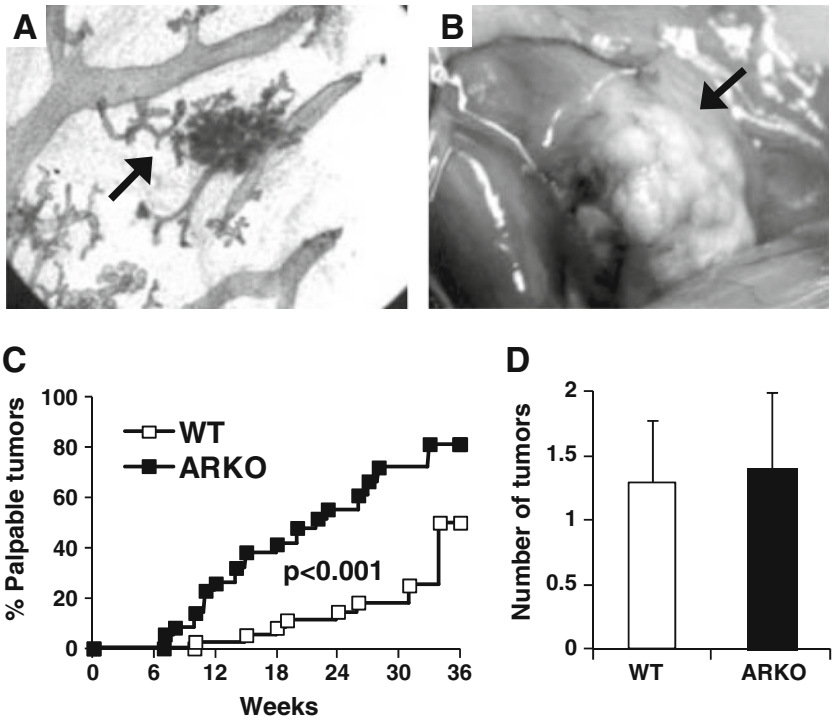

Fig. 1 The DMBA-induced carcinogenesis. (**a**) The DMBA carcinogenesis model generated early gross-lesions detectable at whole mount analysis (*arrow*) and (**b**) palpable mammary tumors. (**c**) Cumulative proportion of ARKO (n=36) and WT (n=39) female mice with palpable mammary tumors within 9 months of observation period. Data are presented as percentage of mice with palpable mammary tumors after the last DMBA dose. Median time to palpable tumor was 34 weeks for WT and 22 weeks for ARKO. The statistical analyses were performed using Kaplan–Mayer survival analysis. (**d**) The number of palpable mammary tumors (per tumor positive mouse) was not significantly different between the WT and the ARKO females (data shown as mean±SD)

Table 1 Mammary cancers in WT and ARKO females

Females	*N*	Median time (IQR)
WT	39	34 (11, n.a.)
WT^{CMV}	19	34 (34, n.a.)
WT^{SOX}	20	>19 (n.a., n.a.)
ARKO	36	22 (12, 33)
$ARKO^{CMV}$	17	20 (12, 28)
$ARKO^{SOX}$	19	27 (15, 33)

Number of mice analyzed (*n*), the median time, and interquartile range in weeks at which 50% of the mice in the group had palpable mammary tumors in WT (combined WT^{Cmv} and WT^{Sox}) and ARKO (combined $ARKO^{Cmv}$ and $ARKO^{Sox}$) females as well as in separated WT^{Cmv}, WT^{Sox}, $ARKO^{Cmv}$, and $ARKO^{Sox}$ females. Statistical analysis by multivariate Cox regression model demonstrated that the susceptibility to mammary cancer was significantly dependent on genotype (WT vs ARKO; $p=0.0024$) but not on line (Cmv vs Sox; $p=0.7186$)

IQR interquartile range (25%, 75%), *na* not achieved

lymph node (mainly on 4th mammary gland) was detected at necropsy exclusively in DMBA-treated males, and histologically confirmed as local lymphomas (Supplemental Fig. 4A, B). The prevalence of mammary gland associated local lymphomas was not different between ARKO (33%; $n=27$) and WT (21%; $n=29$) males (Supplemental Fig. 4C) nor were the results different for $ARKO^{Cmv}$ ($p=0.455$) or $ARKO^{Sox}$ separately ($p=0.120$; data not shown).

All mammary tumors were classified as malignant by a pathologist (GW). Histopathological analysis was performed for all tumors (Fig. 2a–j). The mammary tumors in WT and ARKO females were mainly adenocarcinomas (38% of all mammary tumors; Fig. 2a) or squamous cell carcinomas (scc; 48% of all mammary tumors; Fig. 2f). Remaining tumors were classified as extra-osseous osteosarcoma or mixed adenocarcinoma and osteosarcoma. Most adenocarcinomas were biphasic, containing a layer of CK8-immunopositive luminal epithelium and SMA-positive myoepithelium (Fig. 2b, c). The epithelia were actively proliferating as shown by strong PCNA immunopositivity (Fig. 2d). The scc were characterized by patches of CK8 positive cells with the SMA positive myoepithelial cells surrounding the squamous epithelial layers (Fig. 2g, h). Typically for squamous epithelial differentiation, the basal epithelial layers were highly proliferative (Fig. 2i); 55% of adenocarcinomas and 43% of sccs were positive for ERα protein (Fig. 2. e, j).

DMBA treatment induces ovarian damage. DMBA treatment resulted in ovarian damage in both $ARKO^{Cmv}$ and WT^{Cmv} females evident at the end of experiments (Supplemental Fig. 5A, B). DMBA-treated ovaries were much smaller (1.8±0.3 mg, $n=26$ vs 12±1 mg, $n=8$) that in vehicle oil-treated females. Histological analysis revealed

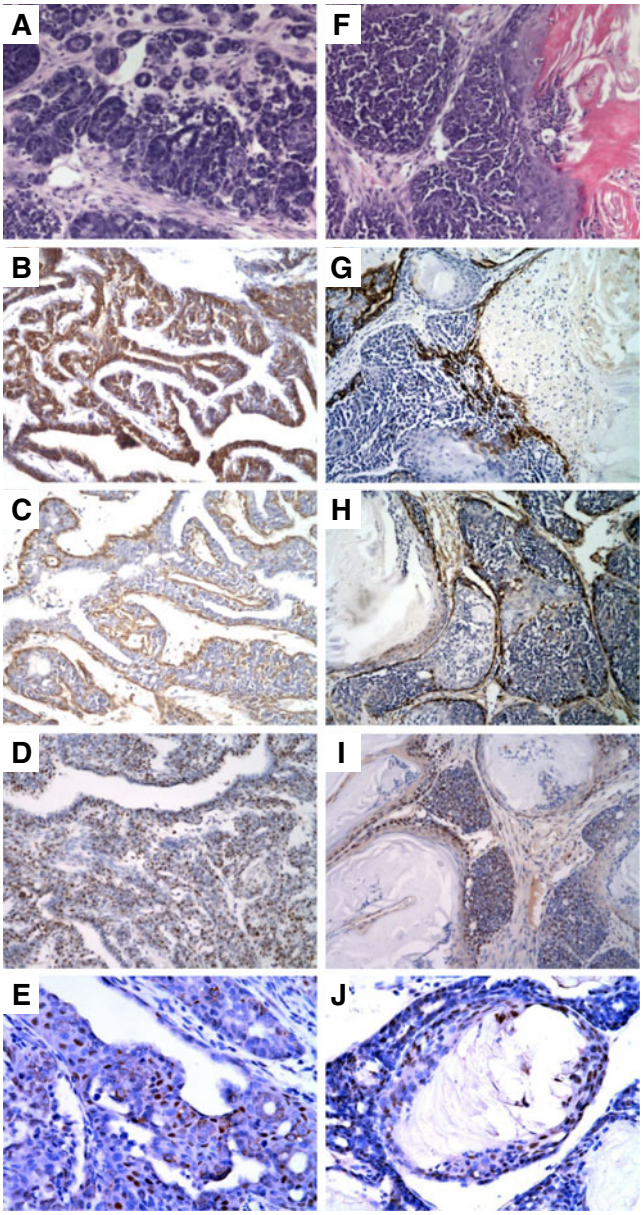

Fig. 2 Histopathology of DMBA-induced tumors. Histopathological analysis was performed for all tumors of all WT and ARKO females (**a**–**h**). The mammary tumors in both WT and ARKO females were mainly adenocarcinomas (38% of all mammary tumors; **a**) or squamous cell carcinomas (scc; 48% of all mammary tumors; **f**). Adenocarcinomas (**a**, H&E) were typically biphasic, containing a layer of CK8-immunopositive luminal epithelium (**b**) and SMA-positive myoepithelium (**c**). Active proliferation was demonstrated by strong PCNA immunopositivity (**d**). Example of ERα immunopositivity (**e**) detected 55% of the adenocarcinomas. The squamous cell carcinomas (**f**; H&E) were characterized by patches of CK8 positive cells (**g**) with the SMA positive myoepithelial cells (**h**). The basal epithelial layers were highly proliferative (**i**). Example of ERα immunopositivity (**j**) detected in 43% of the squamous cell carcinomas

complete loss of follicles and oocytes leading to an afollicular appearance, characteristic of ovarian failure (Supplemental Fig. 5C, D). Ovarian failure was supported by

significantly increased serum FSH ($p<0.001$) and LH ($p=0.008$) in both WT and ARKO females treated with DMBA compared with vehicle oil-treated females (Fig. 3a). Genotype did not significantly influence either serum FSH or LH levels ($p=0.844$, two-way ANOVA) or the elevation induced by DMBA exposure (interaction $p=0.957$; Fig. 3a).

DMBA-treated females displayed reduced serum T ($p=0.015$) and DHT ($p<0.001$) levels in both WT and ARKO females when compared to vehicle oil-treated controls (Fig. 3b). Serum E2 levels were below the LOQ (<5 pg/ml) of LC-MS/MS method in all mice regardless of treatment or genotype. This is in accordance with recent studies reporting very low and mainly undetectable circulating E2 in virgin female mice as demonstrated by sensitive and highly specific tandem MS based methods [26, 53, 85]. Whole mount analysis of mammary glands from both ARKO and WT females showed a markedly reduced ductal tertiary side-branching (Supplemental Fig. 5E, F).

AR suppresses epithelial branching in virgin females and ductal extension in males. Due to similar tumorigenesis in both ARKO lines, further analysis in intact virgin females was performed between WT^{Cmv} and $ARKO^{Cmv}$ mouse lines. To explore the mechanisms leading to the increased susceptibility of ARKO females to mammary tumorigenesis, virgin $ARKO^{Cmv}$ and WT^{Cmv} mammary glands were analyzed at 8 weeks of age (prior to DMBA treatments). Epithelial branching (defined as the number of epithelial branch points per specified area) was non-significantly ($p=0.08$) increased in $ARKO^{Cmv}$ mammary glands when compared to WT (Fig. 4a–c). However, ductal distance (from lymph node to furthest end of the ductal tree) was similar between WT^{Cmv} (13.2±0.3 mm) and $ARKO^{Cmv}$ females (12.7±0.6 mm). In $ARKO^{Cmv}$ males, AR inactivation allowed substantial ductal extension while WT^{Cmv} males displayed only rudimentary epithelial branches (Supplemental Fig. 4) or complete regression of epithelial structures proximal to the lymph node of the 4th inguinal mammary gland (not shown). However, the mammary ducts in $ARKO^{Cmv}$ males lacked the secondary and tertiary branching observed in females (Supplemental Fig. 4).

AR mediates inhibition of epithelial proliferation in virgin mammary glands. Mammary epithelial proliferation was measured by the proportion of PCNA positive cells in intact female $ARKO^{Cmv}$ and WT^{Cmv} mammary glands. Epithelial proliferation was significantly ($p<0.05$) higher in $ARKO^{Cmv}$ when compared to WT^{Cmv} epithelia (Fig. 4d). This was accompanied with significantly increased CyclinE1 ($p=0.048$) mRNA expression in ARKO glands (Fig. 4e) whereas mRNA expression of cell cycle factors CyclinD1 or p21 was not affected (Fig. 4e). Untreated glands had unaffected expression of PR (Fig. 4f, g) and ERα (Fig. 4h, i) at mRNA and protein levels.

Discussion

Clinical evidence implying a role for androgens in breast cancer includes the findings that AR is expressed in 70–90% of breast cancers, most commonly in luminal A and B invasive breast cancers, but also in basal-like cancers [11,

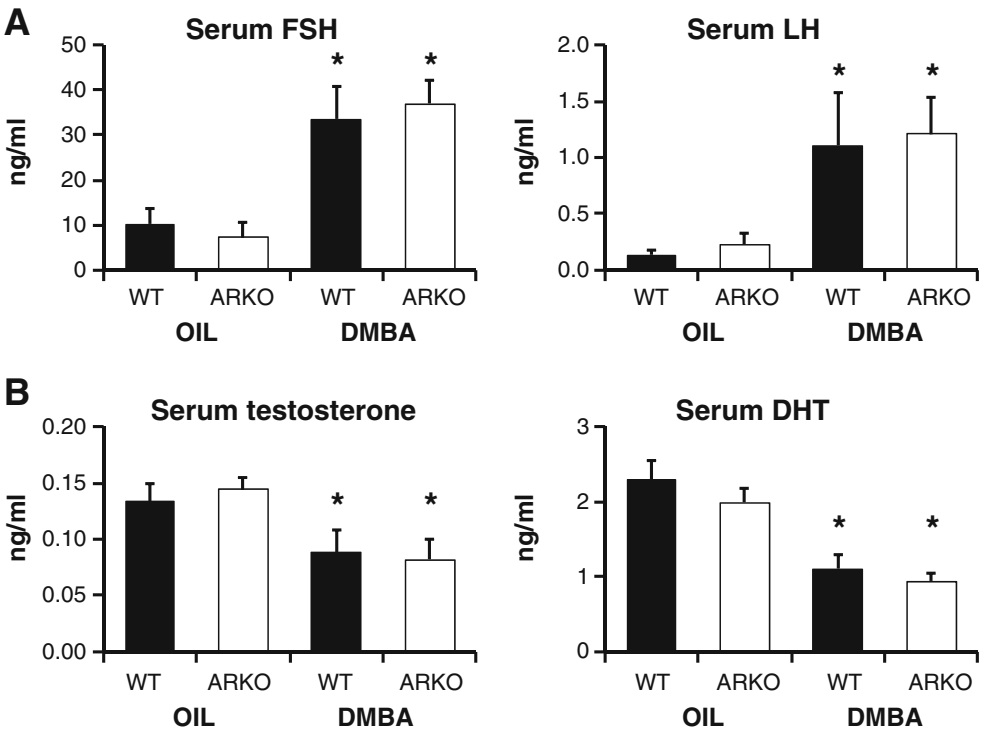

Fig. 3 DMBA influence on serum FSH, LH, testosterone and DHT. DMBA treatment significantly increased serum levels of FSH and LH (**a**) and reduced the serum testosterone and DHT (**b**) in both WT and ARKO females. The genotype did not significantly influence serum levels of gonadotrophins, testosterone or DHT. Data expressed as mean±SE, $n\geq5$. *Asterisk* indicates statistically significant difference ($p<0.05$) from respective oil-treated females

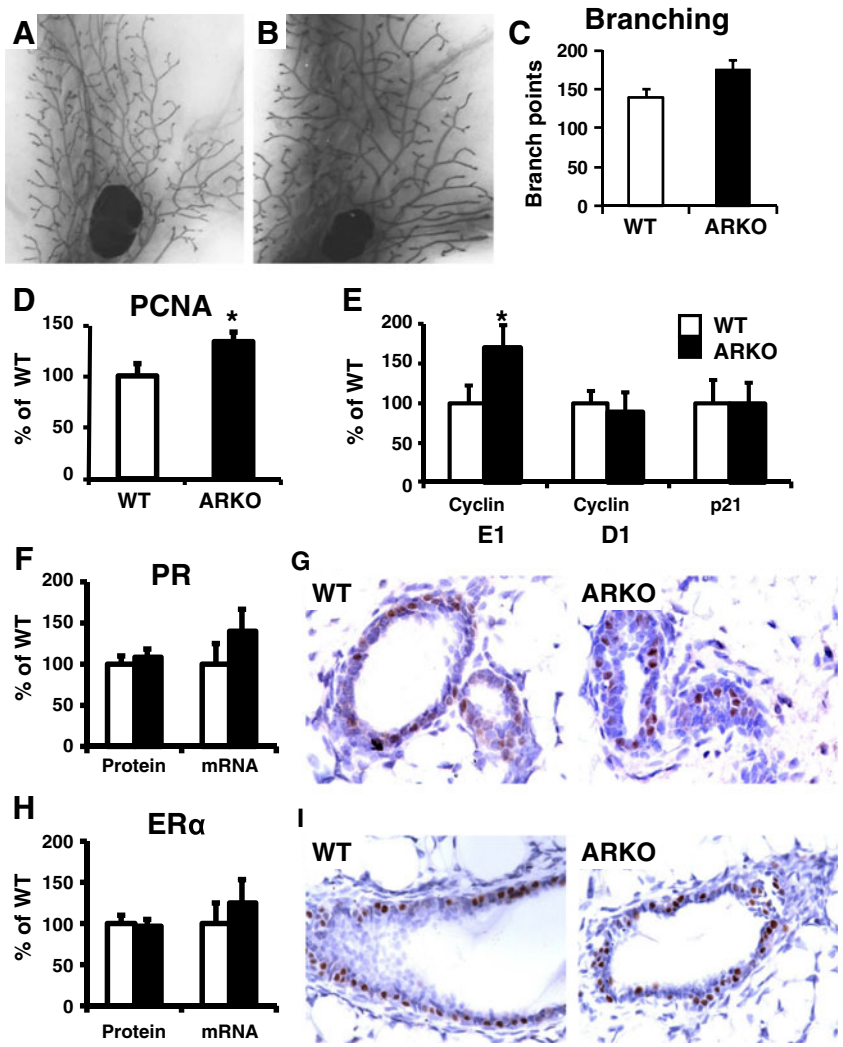

Fig. 4 Comparison of mammary glands between untreated WT and ARKO females at 8 weeks of age (before DMBA administration). The epithelial branching was non-significantly ($p=0.08$) increased in ARKO mammary glands compared to WT at estrus as analyzed by whole mount carmine alum stained glands. Representative photos of epithelial branching in WT (**a**) and ARKO (**b**) females and quantification of epithelial branching points (**c**) are shown. The epithelial proliferation (**d**; % of PCNA positive epithelial cells by stereology) and expression of CyclinE1 (**e**) was significantly increased in ARKO mammary gland compared to WT. mRNA expression of CyclinD1 and cyclin-dependent kinase inhibitor $p21^{Waf1}$ were unaffected (**e**). The % of epithelial cells immunopositive for PR (**f**, **g**) and ERα (**h**, **i**) and the mRNA expression of PR (**f**) and ERα (**h**) were similar between WT and ARKO mammary glands. Data expressed as % of WT and values are mean±SE, $n=6$. *Asterisk* indicates statistically significant difference ($p<0.05$) from WT

27, 43, 57, 59] and androgens as well as androgen-converting enzymes are present in the normal and malignant breast [82–84]. Yet, the role of androgens in breast cancer remains controversial due to the lack of suitable experimental models and ethical constraints on human experimentation. This study provides in vivo evidence that AR is involved in the suppression of susceptibility to DMBA-induced experimental mammary tumorigenesis independent of the mouse genetic background. We demonstrate that androgen-resistant (homozygous ARKO) female mice have significantly faster onset of DMBA-induced mammary tumors compared with WT females.

While our findings are supported by previous reports that androgens inhibit the growth of AR-positive breast cancer cells in vitro [3, 25] and DMBA-induced mammary tumor induction in rats in vivo [23], they contrast with other previous finding that treatment with anti-androgen flutamide alone inhibited growth of DMBA-induced rat mammary tumors [6]. However, this may also reflect the ability of anti-androgens to act as weak androgen agonists when background endogenous androgen activity is low [96]. These ambiguous previous findings emphasize the limitations of pharmacological experiments as some androgen may be aromatized or metabolized to other steroid metabolites with diverse effects. Thus, the present study using genetic mechanisms to block AR-mediated androgen action provides clear evidence that AR-mediated androgen action inhibits experimental carcinogen-induced mammary tumorigenesis in vivo. While caution is needed in extrapolating findings on experimental carcinogenesis in mice to human disease [2], comparative studies demonstrate that DMBA induced mammary tumors are similar to human breast cancers in terms of long relative latency, histotypes, and responses to hormonal interventions [70].

DMBA treatment did not induce epithelial mammary tumors in ARKO males indicating that AR inactivation alone does not predispose to mammary tumors. The lack of DMBA-induced mammary tumors in WT and ARKO males suggests that exposure to normal levels of the ovarian hormones is required during development and/or at the time

of DMBA exposure for emergence of DMBA-induced mammary cancer detected in both WT and ARKO females. This is in accordance with previous studies showing that ovarian grafts are required for high incidence of DMBA-induced mammary tumors in castrated male rats [13]. Similarly, in men AR mutations leading to complete or partial androgen insensitivity are linked to increased breast cancer risk, but typically involve increased exposure to estrogen action [68]. Our ARKO model features a non-functional AR because the deletion of exon 3 eliminates the second zinc finger of the DNA binding domain of the AR protein so that AR is unable to bind to cognate DNA response elements. This engineered mutation replicates the naturally occurring deletion of this exon in humans which causing complete androgen insensitivity in multiple unrelated individuals [65]. Similarly, genetic male mice featuring this exon 3 deleted AR display the classical androgen-resistant tfm phenotype with small abdominal testes, lack of accessory sex organs and female external phenotype [58]. Importantly, the same exon 3 deleted AR variant has been reported as a somatic mutation in women with breast cancer [95] where the loss of androgen inhibition within the breast could have enhanced progression of the cancer. However, AR inactivation alone without DMBA-treatment in females did not induce any spontaneous mammary tumors.

A role for AR in suppression of human breast cancer progression is supported by findings that women with AR-negative breast cancer respond poorly to hormone therapy with reduced overall survival [46, 73], while AR positive cancers are related to smaller tumor size and lack of lymph node metastasis [28]. AR mediated androgen signaling is assumed inhibitory in ERα positive breast cancer [35, 60, 62] and AR may interact directly with estrogen responsive elements [62, 67], consistent with other experimental evidence postulating the existence of functional AR:ERα heterodimers [1]. Based on this, it is proposed that AR may also modulate the response to hormonal therapies targeting ER signaling [15]. This inhibitory role of androgens in ERα positive breast cancers is demonstrated by the clinical use of androgen fluoxymesterone in the treatment of breast cancer [38, 75].

Aromatase inhibitors, together with ER blockers, are currently used as modern adjuvant form of therapy for breast cancer. However, in contrast to the ER blockers, the use of aromatase inhibitors can result in increased circulating androgen levels by blocking the testosterone conversion to E2 [69]. In turn, the increased androgens in the presence of AR are suggested to activate the oncogenic [56] as well as ERα-mediated signaling pathways in ERα negative tumors [67]. Therefore, the AR inhibition is considered as a potential therapeutic target for molecular apocrine breast cancers [19] and a clinical trial of androgen blockage in ER/PR negative, but AR positive breast cancer is underway (ClinicalTrials.gov identifier: NCT00468715). These findings have led to re-consideration of AR as an emerging clinical biomarker with potential as a prognostic marker together with ER, PR, and HER-2 [9, 24] as well as a potential novel therapeutic target [56]. In the present study, both ERα positive and negative mammary tumors were generated by the DMBA treatment providing a useful model for future work to explore the mechanisms and therapeutic options for tumors with differing ERα/AR expression pattern.

Androgens are suggested to inhibit proliferation in vitro [21] and estrogen-induced proliferation of mammary epithelial cells and mammary gland differentiation in vivo [18, 80]. However, the growth suppressive effect of androgens was shown to be age dependent with major effect during the pubertal growth [64]. This is supported by the analysis of virgin, untreated mammary glands in the present study, as the mature ARKO mammary glands displayed increased epithelial proliferation but normal structural development at the time of the DMBA treatment. Hence, our data suggests that AR inactivation increases epithelial proliferation and therefore could sensitize the glands to DMBA-induced DNA damage which underlay the enhanced susceptibility to DMBA-induced mammary tumorigenesis. Furthermore, while the PR and ERα expression in the mammary gland (present study) as well as serum hormone levels at diestrus in untreated ARKO females was similar to the WT, the ARKO females display atypical estrus cycles [88, 89], implying that the AR inactivation may alter the dynamic cycle of circulating and glandular hormones. The resulting hormonal imbalance may influence susceptibility to mammary tumorigenesis in ARKO females; however the precise mechanisms are unclear and warrants further analysis. Yet, no ovarian failure is detected in the untreated, intact ARKO females and despite reduced fertility they remain fertile and are not E2 deficient [88]. Finally, while the epithelial AR expression in the mammary gland suggest a role for epithelial AR, further mechanistic information on the AR role specificly in the mammary gland and in the stroma/epithelia could be gained using mammary epithelial specific knockout models accessible using Cre/loxP techniques [87].

Unlike our ARKO model based on expression of a minimally truncated but non-functional AR protein, another ARKO model completely lacking the AR protein appear to qualitatively have impaired mature mammary development [93]. If differences in mammary phenotype exist, this may indicate an undefined AR activity, independent of direct DNA-binding mediated transactivation present in the exon 3 ARKO model with only minimally truncated AR protein. Similarly, differences in female ARKO models have been observed in ovarian follicle development (reviewed in [90]). Yet, all ARKO males, despite the lack or presence of AR protein, have typical tfm phenotype [51, 58, 94]. This suggests that these undefined AR actions may be related to eg

interactions with steroid hormones/steroid hormone receptors/co-activators important for female physiology and less evident in the highly androgen dependent physiology of males [62].

The strong estrogen dependency of the mammary gland [34] may explain why the significant inhibition of mammary ductal extension was detected in untreated sexually mature males, but not in female mice consistent with their marked gender dichotomy in circulating E2 levels. Without the inhibition of androgens via AR, the male mammary glands may respond to lower levels of estrogens allowing the ductal extension, but do not support the female-type branching morphogenesis. On the contrary, in mature females E2 and progesterone are a major determinant with the AR signaling having only a minor inhibitory role. This demonstrates that the level of mammary growth inhibition exerted by AR mediated signaling depends on the balance between the inhibitory androgenic and stimulatory estrogenic signals.

DMBA-induced ovarian toxicity involves destruction of all follicle types in the ovary as observed in cell cultures [37] and in vivo [7, 91] but the hormonal impact of this upon DMBA-induced experimental breast cancer has been little studied. Our findings of marked increases in serum LH and FSH, consistent with ovarian ablation, extend previous morphological findings reported in rats [42]. Although the highly sensitive LC-MS/MS method was unable to measure serum E2, the decrease in serum T implies estrogen deficiency as T is the obligate steroidogenic precursor of E2 and this is further supported by the striking increases in serum LH and FSH and the atrophied mammary epithelia of DMBA-treated females. As the DMBA-induced ovarian damage occurred equally in both WT and ARKO females, it is unlikely that the ovarian damage alone can explain the increased susceptibility of ARKO females to DMBA-induced mammary tumorigenesis. However, the implication of DMBA-induced estrogen deficiency combined with greater susceptibility to estrogen dependent mammary tumorigenesis in ARKO females indicate suppression of estrogen sensitivity as a possible mechanism of AR functions in the mammary gland [62]. In addition, the greater susceptibility to DMBA induced ovarian damage in mice compared with rats, as suggested by in vivo toxicity studies [7, 52] and ex vivo culture system [37], may explain the correspondingly greater in vivo susceptibility of rats to estrogen dependent DMBA-induced mammary tumors.

Conclusions

In conclusion, our results provide unambiguous genetic evidence characterizing a role for AR-mediated androgen actions as tumor suppressors in experimental breast cancer. The present findings create a novel experimental model in which to test the AR role in breast cancer pathogenesis such as the suggestion, for example, that the benefits of medroxyprogesterone acetate in breast cancer treatment may be due to its activation of AR rather than PR mechanisms [4]. The results have important implications on the treatment strategies of human breast cancer and presents evidence supporting further evaluation of the involvement of and targeting of AR to improve endocrine therapy at all stages of human breast cancer.

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Conflict of interests The author(s) declare that they have no conflict of interest.

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