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ORIGINAL ARTICLE

Androgen Receptor Actions Modify Skin Structure and Chemical Carcinogen-induced Skin Cancer Susceptibility in Mice

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Abstract Men are significantly more susceptible to non-melanoma skin cancers than women, and the androgen receptor (AR) is widely distributed in the skin, suggesting a ro\le for androgens acting via AR. Therefore, we explored the role of androgen action via AR in susceptibility to experimental 7,12-dimethylbenz[a]anthracene (DMBA)-induced skin carcinogenesis and in skin structural development of male and female mice. We demonstrate that both the male gender and androgen action via AR modify the susceptibility to carcinogen-induced skin cancer, but the effect depends on the carcinogenesis model used. Following systemic DMBA exposure, males were significantly ($p<0.05$) more susceptible to DMBA-induced experimental skin cancer than females and AR inactivation significantly delayed cancer detection in both male (median time to palpable tumours 19 vs. >35 weeks (wild-type [WT] vs. AR knockout [ARKO], $p<0.001$) and female (27 vs. >35 weeks, $p=0.008$)) mice. In contrast, following DMBA/12-O-tetradecanoylphorbol-13-acetate (TPA)-induced multistage local skin carcinogenesis, AR inactivation protected against formation of DMBA-induced skin cancers in both male and female mice. The skin structure was also affected by gender effect as well as the AR inactivation and could at least partly explain the different responses between the carcinogenesis models (systemic vs. topical). In addition, AR inactivation modified *Cox-1* and *Cox-2* expression in the skin, suggesting possible molecular mechanism for the AR effect on skin. Finally, some gender differences are observed also in ARKO mice insensitive to androgens, suggesting that factors other than androgens also play a role in gender-dependent skin carcinogenesis.

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Introduction

Skin is made up of three basic layers: the epidermis, dermis and hypodermis. The epidermis is a relatively thin outer layer of stratified squamous epithelium composed of mainly keratinocytes, melanocytes, Langerhan's cells and Merkel cells. The dermis is a thick layer of fibrous and elastic tissue made up of collagen, elastin and fibrillin produced from fibroblasts, the main cell type. The dermis also contains hair follicles, blood vessels, nerve endings and sweat and sebaceous glands. The hypodermis is the bottom layer of the skin and is mainly composed of adipose tissue.

Gender differences in the skin structure are well known and attributed to differences in exposure to endogenous androgens and estrogens. Males have thicker skin in general, with 190 % thicker dermis observed in male compared with female mice [2]. Yet, the epidermis and hypodermis are thinner in males than in females. Conversely, skin collagen content is significantly greater in mature male compared with mature female mice [16]. Epidemiological studies also suggest significantly more non-melanoma skin cancers occurring in men than in women [23], with men also having greater mortality from melanoma [7]. While this has been attributed to gender behavioural differences in sun exposure, recent experimental

studies also reveal significant gender differences in UVB-induced skin cancer susceptibility with male mice being at greater risk than female mice [24]. This male gender predisposition is also supported by males being more susceptible to chemical carcinogen-induced skin tumours [13].

Androgens have a significant role in structural development of male mouse skin suggesting a hormonal basis for the evident gender differences. Androgen receptor (AR) is widely expressed throughout the skin layers [28], suggesting tissue-specific effects of androgens are exerted via AR expressed in the skin. Testicular feminized (tfm) male mice lacking functional AR due to an inactivating mutation in the *Ar* gene have less dermal collagen when compared with wild-type (WT) males [16]. Yet, the role of androgen action via AR in females with regard to skin structural development as well as in experimental skin carcinogenesis is not well understood.

The aim of the present study was to determine the role of androgen action via AR in susceptibility to experimental 7,12-dimethylbenz[a]anthracene (DMBA)-induced skin carcinogenesis and in skin structural development of male and female mice. We demonstrate that both the male gender and androgen action via AR modify the susceptibility to carcinogen-induced skin cancer, but the effect depends on the carcinogenesis model used. Similarly, the gender effect as well as the role of AR in formation of skin structure could at least partly explain the different responses between the carcinogenesis models (systemic vs. topical). Finally, gender differences are observed also in mice insensitive to androgens (AR knockout, ARKO), suggesting that factors other than androgens also play a role in gender-dependent skin carcinogenesis.

Methods

Generation of ARKO mice ARKO^{Cmv} (C57Bl) and ARKO^{Sox} (FVB/N) female and male mice homozygous for global AR inactivation were generated using Cre/loxP system and genotyped as previously described [25, 21]. Cre/loxP system was utilized as ARKO females cannot be produced by natural breeding. Wild-type (WT) females and males from the respective colonies WT^{Cmv} and WT^{Sox} were used as controls. The AR inactivation in the global ARKO^{Cmv} and ARKO^{Sox} mice was confirmed by development of *tfm* phenotype in ARKO^{Cmv} and ARKO^{Sox} males [21].
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 To determine the influence of AR inactivation on skin structure at the time of DMBA exposure, skin samples were collected from intact, untreated 5- and 8-week-old WT^{Sox} and ARKO^{Sox} male and female mice.

To determine the effect of gender and androgen actions via the AR on skin cancer susceptibility, females (n=18 for WT^{Cmv}, 20 for WT^{Sox}, 17 for ARKO^{Cmv} and 17 for ARKO^{Sox}) and males (n=16 for WT^{Cmv}, 16 for WT^{Sox}, 13 for ARKO^{Cmv} and 16 for ARKO^{Sox}) 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. 1a) as previously described. Mice were examined weekly for palpable mammary tumours and sacrificed at 9 months after DMBA treatment, unless the tumours reached ~1 cm in diameter or mice were moribund at an earlier age in which case they were then sacrificed for ethical reasons. 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}).

As ARKO^{Cmv} and ARKO^{Sox} female and male mice responded similarly to systemic DMBA exposure (see data), only ARKO^{Sox} mice (and respective WT^{Sox}) were used in the following experiments. In addition to the skin cancers, systemic DMBA exposure induces lymphomas and breast cancers [21]. Therefore, we wanted to explore the AR effects on the skin cancer susceptibility by a commonly used two-stage chemical skin carcinogenesis model [1]. The two-stage model includes cancer initiation with the carcinogen DMBA, and the tumour development is elicited with the tumour-promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA). WT and ARKO males (n=14 and 11, respectively) and females (n=11 and 9, respectively) were shaved at 7 weeks of age and exposed to a single dose of 40 μM DMBA (100 μl in DMSO), followed by twice weekly treatment with 50 μM TPA (100 μl in DMSO) for 15 weeks (Supplemental Fig. 1b). The appearance and number of skin papillomas were examined weekly for 28 weeks after the DMBA exposure. The cumulative papilloma number was determined by successively adding the tumours that developed each week.

To explore the possible effect of AR inactivation on expression of DMBA-induced genes in the skin, the WT and ARKO males (six for WT^{Sox} and six for ARKO^{Sox}) were exposed to a single dose of 1 mg DMBA (in 100 μl sesame oil vehicle) delivered by gavage and skin samples were collected 3 days after dosing.

Tissue collection Mice were anaesthesized (Ketamin/Xylasil) and terminated by cardiac bleeding. In experiments 1 and 2, skin papillomas were counted and fixed overnight in 4 % paraformaldehyde at +4 °C. In experiments 3 and 4, the dorsal skin was shaved and either fixed overnight in 4 % paraformaldehyde at +4 °C or snap frozen in liquid nitrogen.

Confirmation of mutated Ar expression To confirm the presence of inactivated *Ar* in the skin, the expression of mutated, exon 3-deleted *Ar* or intact *Ar* in the mammary glands was

determined by reverse transcription polymerase chain reaction (RT-PCR) as previously described [20]. Exclusively exon 3-deleted Ar product was confirmed in 8-week-old ARKO skin while only the larger wild-type *Ar* product with intact exon 3 was detected in WT skin (Supplemental Fig. 1c, d).

Histopathological analysis, assessment of skin thickness and collagen layer Paraffin-embedded, fixed tissues were sectioned to 5 μm. For structural analysis, the sections were stained using haematoxylin and eosin (H&E), and dermal and epidermal thickness was measured using CASTGRID V1.10 stereology software. CASTGRID was used to create a random point grid over the skin cross-section and thickness measured at the points overlapping either dermis or epidermis perpendicular to the bordering layers. For the dermis and epidermis separately, at least five measurements were obtained for each individual sample. The same method was used for analysis of the thickness of collagen layer from Masson's trichrome-stained sections. To assess dermal collagen deposition, Masson's trichrome staining was used to detect stain collagen fibres in blue. Collagen density was quantified via converting Masson's trichrome-stained skin section images to binary format using ImageJ software, where the collagen fibres appeared black. The percentage of the black fibres relative to the total dermis area in each image was defined as percent collagen density.

mRNA expression mRNA extraction, cDNA conversion and real-time RT-PCR were performed as previously described [22]. The following primers were used for analysis of cyclooxygenase 1 (Cox-1) and cyclooxygenase 2 (Cox-2) expression: Cox-1-forward (5′-ACCTACGTCTACGCCAAAGG-3′) and Cox-1-reverse (5′-GTGGTTTCCAACCAAGATCA-3′) and Cox-2-forward (5′-CCGTGCTGCTCTGTCTTAAC-3′) and Cox-2-reverse (5′-TTGGGAACCCTTCTTTGTTC-3′). Cyclophilin and Rpl19 were used as reference genes as previously described [20, 25].

Data analysis and statistics Data are shown as mean±SE unless otherwise stated. The statistical analyses were performed using Kaplan-Meier survival analysis or analysis of variance (ANOVA) (SPSS Inc., Chicago, IL). *p* values less than 0.05 were considered statistically significant.

Results

Androgens acting via AR modify skin thickness To determine the effect of AR inactivation on skin structural development, the skin dermal and epidermal thickness was compared between WT and ARKO males and females. At 8 weeks of age, the dermis thickness was significantly (two-way ANOVA; $p<0.001$) affected by both the AR inactivation and the gender. WT males had significantly thicker (48 % thicker) dermis than ARKO males (Fig. 1a; Supplemental Fig. 2a). Similarly, in females, the dermis thickness was significantly greater (25 % thicker) in WT females when compared to ARKO females (Fig. 1a; Supplemental Fig. 2a). The dermis thickness was also affected by gender and was significantly greater (48 % thicker) not only in WT males when compared to WT females but also in ARKO males when compared to ARKO females (25 % thicker). A similar pattern was observed at 5 weeks of age (data not shown). At 8 weeks of age, epidermal thickness was significantly reduced by AR inactivation (two-way ANOVA, $p<0.001$). There was also a trend for a gender effect (two-way ANOVA; $p=0.06$) with males having thicker epidermis than females (Fig. 1b; Supplemental Fig. 2a).

Androgens via AR modify thickness of collagen layer but not the collagen density of the skin To determine the AR-mediated androgenic regulation of collagen deposition in the skin, the skin sections from WT and ARKO males and females were stained with Masson's trichrome staining (Supplemental Fig. 2b) to visualize the collagen layer. The collagen density in the specified area was significantly dependent on the AR status in both males ($p=0.001$) and females ($p=0.003$) at the age of 5 weeks (Fig. 2a). In addition, the collagen density in the specified area was greater ($p=0.028$) in WT males than in WT females (Fig. 2a). However, at 8 weeks of age, the collagen density in the specified area was not significantly affected by gender or AR status (Fig. 2b). However, the thickness of collagen layer in the skin, as analysed using CASTGRID V1.10 stereology software, was significantly ($p=0.01$) influenced by the AR inactivation in both males and females (Fig. 2c, d). This was evident already at 5 weeks of age, and the difference remained in 8 weeks of age.

AR inactivation protected against formation of systemic DMBA-induced skin cancers in both male and female mice We first determined the effect of AR inactivation on the susceptibility to carcinogen-induced skin cancers following systemic DMBA treatment. The development of skin pathology was compared between WT^{Cmv}, WT^{Sox}, $ARKO^{Cmv}$ and $ARKO^{Sox}$ males and females following six weekly doses of 1 mg DMBA. The effect of AR inactivation on skin cancer incidence was not affected by the genetic background of the mice. The emergence of DMBA-induced skin papillomas did not significantly differ between WT^{Cmv} and WT^{Sox} males or females or between $ARKO^{Cmv}$ and $ARKO^{Sox}$ males or females (Table 1; Supplemental Fig. 3), and therefore, the appearance of skin papillomas between WT and ARKO was determined as combined data for ARKO ($ARKO^{Cmv}$ and $ARKO^{Sox}$) and WT (WT^{Cmv} and WT^{Sox}).

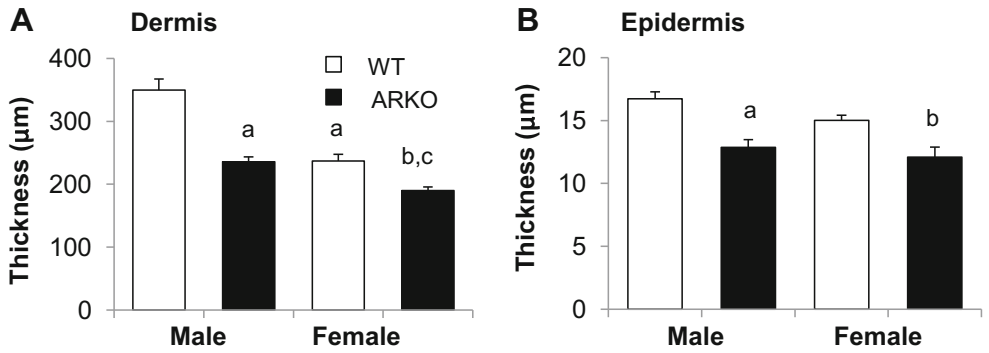

Fig. 1 Dermis (**a**) and epidermis (**b**) thickness (μm) in WT and ARKO male and female mice. The dermal thickness of the skin was dependent on both the gender ($p<0.05$) and the AR status ($p<0.05$) while the epidermal thickness was only affected by AR status ($p<0.05$) but not by gender (two-way ANOVA). Data shown as mean±SE; $N=9$ and 8 for WT male and female and 10 and 9 for ARKO male and female. *a*, significantly different from WT male; *b*, significantly different from WT female; *c*, significantly different from ARKO male

When compared to WT males, the onset of skin cancers was significantly ($p<0.001$; Mantel-Cox test, Kaplan-Meier) delayed in WT and ARKO females and by AR inactivation in ARKO males (Fig. 3a). Similarly, AR inactivation significantly delayed the appearance of skin cancers in ARKO females when compared to WT females ($p=0.008$) (Fig. 3a). While the onset of tumours was similar between WT females and ARKO males (Table 1), the cumulative skin cancer incidence at 9 months was reduced by AR inactivation both in females (9±6 % [mean±SE]; $n=23$) and in males (57±10 %; $n=27$) compared with the incidence in WT females (88±8 %; $n=37$) and WT males (92±7 %; $n=29$) (Fig. 3a).

The total number of papillomas developed in 36 weeks was compared among the genotypes and genders. Both gender ($p=0.009$) and genotype ($p<0.001$) had a significant effect on papilloma numbers (two-way ANOVA). With males having significantly more papillomas compared to females in WT (1.8±0.4 vs. 1.0±0.2 papillomas) and in ARKO (0.6±0.2 vs. 0.2±0.1 papillomas) mice (Fig. 3b). The skin cancers were mainly papillomas lined by hyperkeratotic, stratified squamous epithelium. Few sebaceous hyperplasias were also detected. Five random papillomas from each genotype and gender were stained with H & E and graded by a pathologist to assess any differences between the genotypes. Most of the papillomas were graded as squamous papillomas (Fig. 3c) and

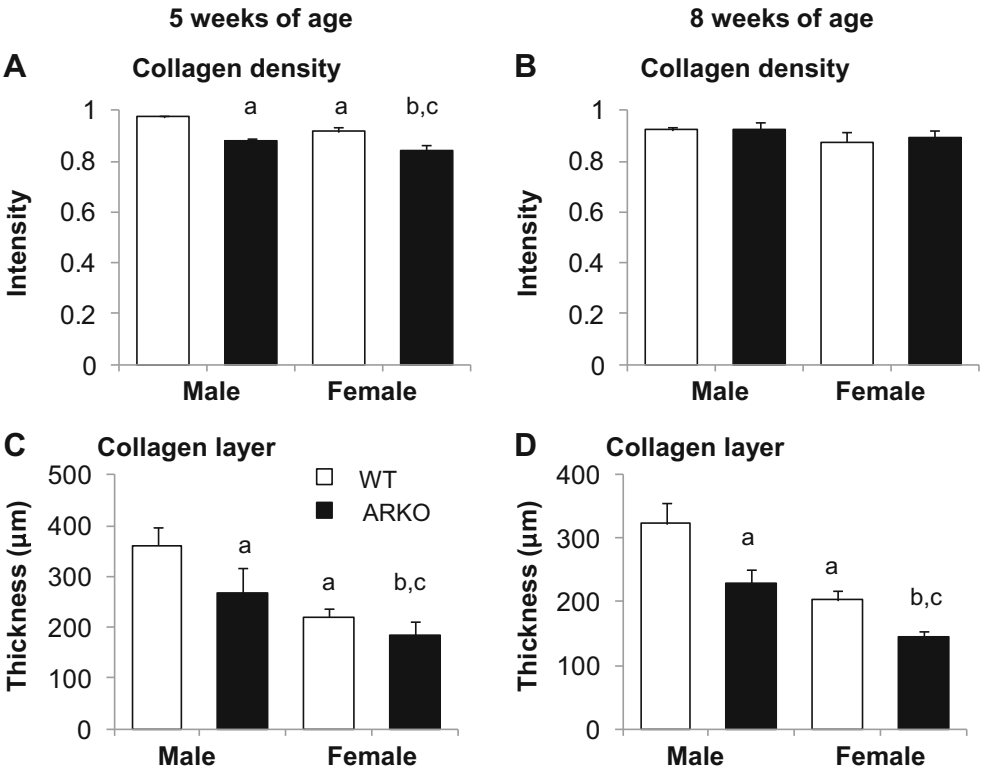

Fig. 2 Collagen density within collagen layer (**a**, **b**) and thickness (μm) of the collagen layer (**c**, **d**) in WT and ARKO male and female mice at 5 weeks (**a**, **c**) and 8 weeks (**b**, **d**) of age. At 5 weeks of age, the collagen density was significantly dependent on the AR status ($p<0.01$) and gender ($p<0.05$). However, at 8 weeks of age, the collagen density was not significantly affected by gender or AR status. However, the thickness of collagen layer in the skin was significantly influenced by the AR status ($p<0.05$) and gender ($p<0.05$) at 5 and 8 weeks of age (two-way ANOVA). Data shown as mean±SE; $N=4$. *a*, significantly different from WT male; *b*, significantly different from WT female; *c*, significantly different from ARKO male

Table 1 Skin cancers in WT and ARKO males and females. The number of mice analysed (*n*), the median time and interquartile range in weeks at which 50 % of the mice in the group had palpable mammary tumours in WT (combined WT^{Cmv} and WT^{Sox}) and ARKO (combined $ARKO^{Cmv}$ and $ARKO^{Sox}$) males and females as well as in separated WT^{Cmv}, WT^{Sox}, $ARKO^{Cmv}$ and $ARKO^{Sox}$ males and females. Statistical analysis by multivariate Cox regression model demonstrated that the susceptibility to skin cancer was significantly dependent on genotype (WT vs. ARKO; $p<0.001$) and sex (male vs. female; $p<0.001$) but not on line (Cmv vs. Sox; $p=0.218$)

Males	*n*	Median time (IQR)
WT	32	19 (12, 22)
WT^{CMV}	16	16 (14, 21)
WT^{SOX}	16	21 (11, 22)
ARKO	29	>40 (25, #)
$ARKO^{CMV}$	13	30 (25, #)
$ARKO^{SOX}$	16	>38 (22, #)
Females	*n*	Median time (IQR)
WT	38	27 (22, 29)
WT^{CMV}	18	26 (23, 29)
WT^{SOX}	20	27 (22.3, #)
ARKO	34	>45 (#, #)
$ARKO^{CMV}$	17	>39 (#, #)
$ARKO^{SOX}$	17	>45 (#, #)

Pound (#) sign means not achieved

IQR interquartile range (25, 75 %)

only a few as sebaceous hyperplasia (Fig. 3d). While sebaceous hyperplasia was only detected in females, no obvious differences between the genotypes (WT vs. ARKO) were found. In addition, AR inactivation alone did not predispose to skin cancers, as ARKO or WT males or females treated with vehicle without DMBA did not develop any tumours (data not shown).

AR inactivation protected against the formation of multistage local DMBA/TPA-induced skin cancers in both male and female mice The effect of AR inactivation on susceptibility to carcinogen-induced skin cancers was also determined following local, the traditional two-stage skin carcinogenesis model. The skin of WT and ARKO male and female mice were exposed to an initiator, DMBA, and a promoter, TPA. In contrast to systemic DMBA treatment, the onset of skin cancers was similar between WT and ARKO males following local, two-stage DMBA/TPA carcinogenesis (Fig. 4a). All mice developed tumours by 20 weeks. The median time for tumour development was 11 weeks for all genotypes, except WT female which was 13 weeks. However, when the cumulative papilloma number was determined through to week 28 (Fig. 4b), the androgen resistance in both genders increased the average cumulative papilloma number. At 20 weeks after DMBA exposure, the total number of papillomas was affected by genotype ($p=0.018$) but not by gender when analysed by two-way ANOVA (Fig. 4c). The ARKO male mice had 1.5 times more papillomas compared to WT males, while in ARKO females had 2.3 times more papillomas than WT females (Fig. 4c).

Similarly to systemic DMBA exposure, four or five papillomas from each genotype within the two-stage carcinogenesis-treated groups were selected and analysed for pathology. All papillomas were found to be squamous papillomas, with only one of each WT and ARKO female papillomas showing early signs of sebaceous hyperplasia (data not shown).

AR inactivation modified Cox-1 and Cox-2 expression in the mouse skin As Cox enzymes were shown to be involved in skin carcinogenesis, may differentially affect the susceptibility to two-stage carcinogenesis model and systemic DMBA-induced carcinogenesis model [19], and can be regulated by androgens [27], we explored the effect of AR inactivation on the expression of *Cox-1* and *Cox-2* (Fig. 5a, b) mRNA in the skin following short-term local DMBA exposure. *Cox-1* mRNA expression (Fig. 5a) in the skin was significantly ($p=0.049$; two-way ANOVA) affected by the local DMBA treatment, and the effect was dependent on genotype ($p=0.035$; two-way ANOVA). Cox-1 mRNA expression was induced by DMBA treatment in WT male skin but not in ARKO (Fig. 5a). The mRNA expression for *Cox-2* (Fig. 5b) was significantly (two-way ANOVA; $p<0.01$) modified by both AR status and treatment. Cox-2 expression was reduced by AR inactivation in ARKO when compared to WT following vehicle or DMBA treatment. In addition, DMBA exposure reduced the Cox-2 expression in both WT and ARKO skin (Fig. 5b).

Discussion

Two in three Australians will be diagnosed with some form of skin cancer by the age of 70 and are four times more likely to develop skin cancer than any other form of cancer [23]. Gender differences are recognized in skin cancer incidence as well as mortality [7] with men being more susceptible than women. This gender difference is supported by the present study demonstrating that male mice are significantly more susceptible to the carcinogen-induced skin cancer when compared to females. Similarly, a recent study found that male mice are also more susceptible to UVB-induced skin carcinogenesis than females [24]. However, the present study is the first to investigate the potential role of androgens in skin cancer susceptibility. We have demonstrated that androgen resistance delays systemic DMBA treatment-induced papilloma development not only in male but also in female mice. The same effect of gender and AR resistance was also

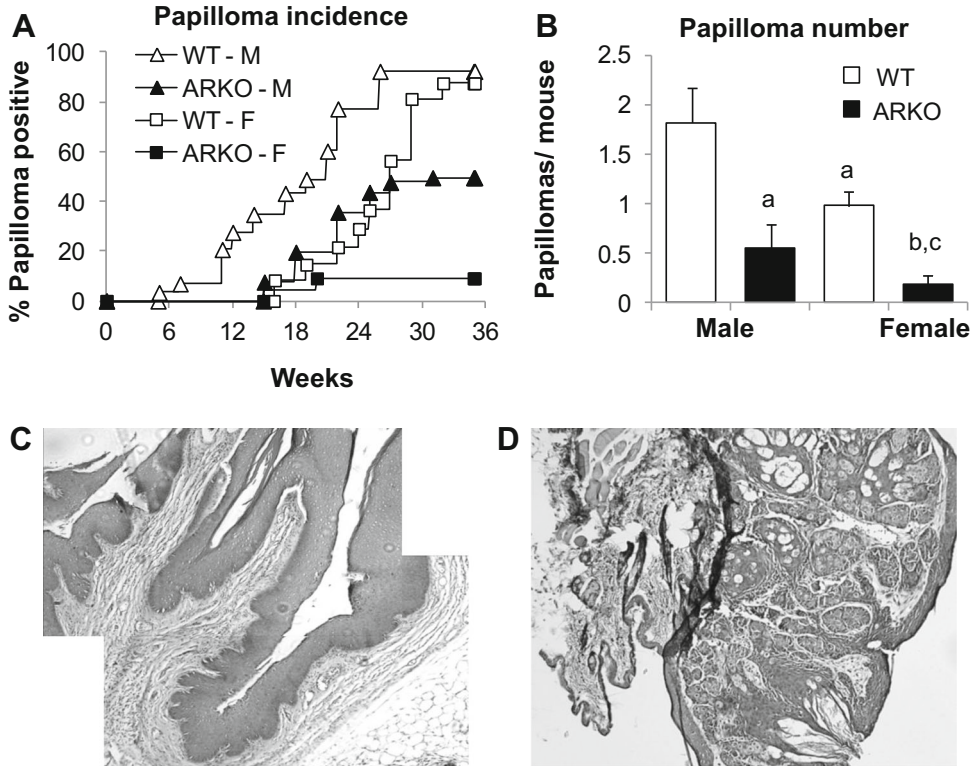

Fig. 3 Cumulative proportion of ARKO (n=29) and WT (n=32) male as well as ARKO (n=34) and WT (n=38) female mice with skin papillomas within 9 months of observation period (**a**) following exposure to six weekly systemic doses of 1 mg DMBA. Data are presented as percentage of mice with skin papillomas after the last systemic DMBA dose. Median time to skin papilloma development was 19 weeks for WT males and 27 weeks for WT females compared to >40 weeks in both ARKO males and females. The statistical analyses were performed using Kaplan-Meier survival analysis. The total number of papillomas developed in 35 weeks (**b**) was dependent on both gender (p<0.01) and genotype (p<0.001) (two-way ANOVA). Representative photos of H & E-stained skin papillomas (**c**, **d**). The skin cancers were mainly papillomas lined by hyperkeratotic, stratified squamous epithelium (**c**) and some sebaceous hyperplasias (**d**). *a*, significantly different from WT male; *b*, significantly different from WT female; *c*, significantly different from ARKO male

demonstrated in the total number of papillomas being significantly greater in WT and male mice compared to their ARKO and female counterparts. This provides one explanation for the known greater susceptibility of males to skin cancer and proposes a significant role of androgens via AR in this gender difference.

The fact that there is a difference between gender not only in the WT subjects but also in the ARKO subjects in both papilloma development rate and number reveals that androgens alone cannot fully explain the gender difference in susceptibility to experimental skin cancer. It is suggested that exposure to estrogens also has a significant role in skin structure as well as in skin pathology [3]. Ovariectomized female mice had increased papilloma incidence [14] supporting a significant role for ovarian hormones in reducing experimental skin cancer susceptibility. This was further supported by reduced experimental skin cancer susceptibility by endogenous estrogens [15]. Therefore, it is suggested that estrogens could explain the gender difference in DMBA-induced skin cancer susceptibility in ARKO male and female mice.

As the systemic DMBA-treatment induces multiple other types of tumours including lymphomas as well as breast and ovarian cancers [21, 26], the effects of AR inactivation on skin cancer susceptibility were also explored following a well-known and widely used two-stage carcinogenesis skin cancer model with local, skin-specific DMBA/TPA treatment. In contrast to the systemic DMBA treatment, the effect of both gender and genotype in the DMBA-induced median time for papilloma development was lost following the two-stage carcinogenesis model. We propose that either the local initiation with the DMBA and/or the promotion by TPA could be so intense that they overcome the effects of gender and AR inactivation on papilloma development, whereas the systemic treatment with DMBA alone could be slow and/or weak enough to remain influenced by the differences in papilloma development between genders and genotypes. The strength of the two-stage skin cancer model is demonstrated by significantly faster development of papillomas when compared to systemic DMBA treatment. The greater intensity of the local DMBA/TPA treatment was also obvious when comparing the mean number of papillomas in mice. It is suggested that the final papilloma number can be used as a measure of initiation, while the rate of papilloma development, as a measure of promotion [12]. This implies that androgen resistance in the

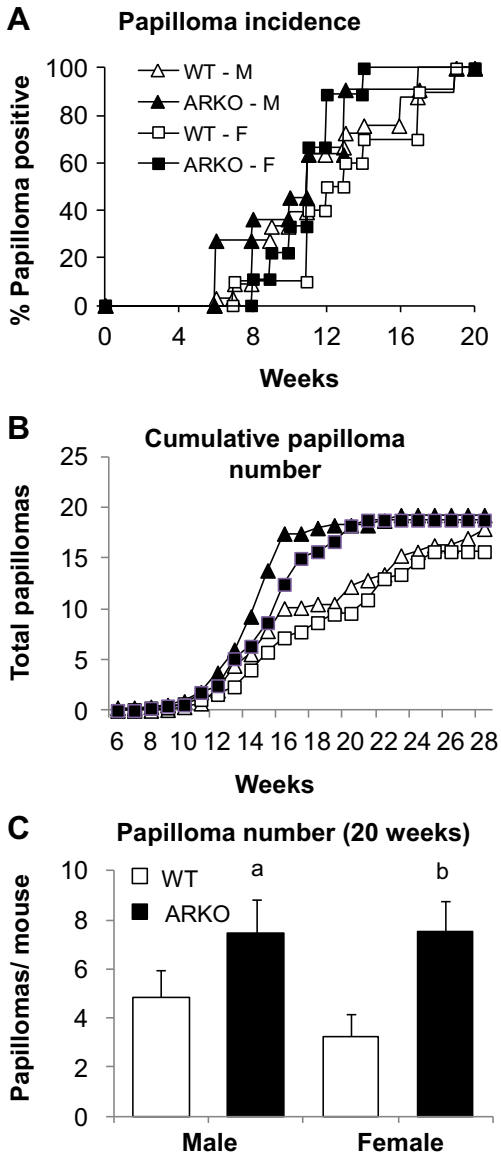

Fig. 4 Cumulative proportion (**a**); percentage of mice with skin papillomas of ARKO (n=11) and WT (n=14) male as well as ARKO (n=9) and WT (n=11) female mice with skin papillomas, cumulative number of skin papillomas (**b**) as well as the average papilloma number per mice at 20 weeks (**c**) following local, the traditional two-stage skin carcinogenesis model. The genotype or the AR status did not affect the median time to papilloma development in the two-stage carcinogenesis model. However, the cumulative papilloma number at 20 weeks after DMBA exposure was affected by genotype (p<0.05) but not by gender (two-way ANOVA). *a*, significantly different from WT male; *b*, significantly different from WT female

two-stage model increases initiation but was without major effect on promotion. Perhaps then the gender differences and androgen functions in systemic treatment act as promotion, and this effect is therefore overcome with the strong promoter, TPA in the two-stage model.

Interestingly, the effect of androgens acting via AR was observed when papilloma development was measured as the cumulative increase papilloma number. Similarly, the total number of papillomas at 18 weeks following initial DMBA treatment was significantly affected by AR inactivation. However, opposite to the systemic treatment where AR inactivation reduced the total number of papillomas, after two-stage carcinogenesis, the AR inactivation increased the total number of papillomas. Therefore, we demonstrated that the local, two-stage carcinogenesis remained at least partly dependent on the AR effect, but the effect was opposite to that found following systemic DMBA. There could be multiple reasons for this difference. We propose that the systemic DMBA- and two-stage DMBA model-induced skin cancers may have different mechanisms of tumorigenesis that could be differentially affected by androgens (and other gender differences). Unfortunately, since systemic DMBA is not often used to specifically look at skin cancer, any differences in mechanisms between the two-stage and DMBA-induced models have not been sufficiently investigated to clarify these issues.

Similar disparity between the DMBA initiation alone (systemic treatment) and the two-stage model for skin carcinogenesis was observed in mice over-expressing COX-2 enzyme. A specific COX-2 inhibitor, celecoxib, interfered with tumour promotion but had no effect on initiation [19]. This supports a difference in the mechanisms of tumorigenesis between the models which could be related to inflammation, as both DMBA and TPA are known to induce inflammation [4]. As the regulation of COX enzymes, similarly to AR inactivation, differentially affected the systemic (initiation only) and local (initiation/promotion) skin carcinogenicity, COX enzymes are associated with development of many cancers [17] and androgens may also regulate the COX enzyme expression [27], we explored the effect of AR inactivation on *Cox-1* and *Cox-2* mRNA expression in the skin following DMBA exposure. We found that AR inactivation significantly modified the DMBA-induced *Cox-1* mRNA expression in the mouse skin. Interestingly, opposite to Cox-1 mRNA expression, the expression of Cox-2 was significantly reduced by DMBA treatment in both WT and ARKO skin when compared to respective vehicle-treated expression. The AR inactivation also significantly modified the Cox-2 expression in the mouse skin with vehicle-treated ARKO males having significantly lower expression when compared to vehicle-treated WT. Based on these findings, Cox signalling could be one of the molecular mechanisms for androgen effects on skin cancer susceptibility. However, the exact cellular events responsible for the Cox effect on tumorigenesis, while supported in epithelial cancers [18, 8], are not entirely clear.

To determine if the androgens also modified the type of papillomas developed after the DMBA or DMBA/TPA exposures or if any papillomas developed into carcinomas, the randomly selected papillomas from each group were histologically analysed by a pathologist. Almost all of the selected papillomas were squamous papillomas, with a very rare occurrence of sebaceous hyperplasia. Of the samples analysed for histopathology, sebaceous hyperplasia was only detected

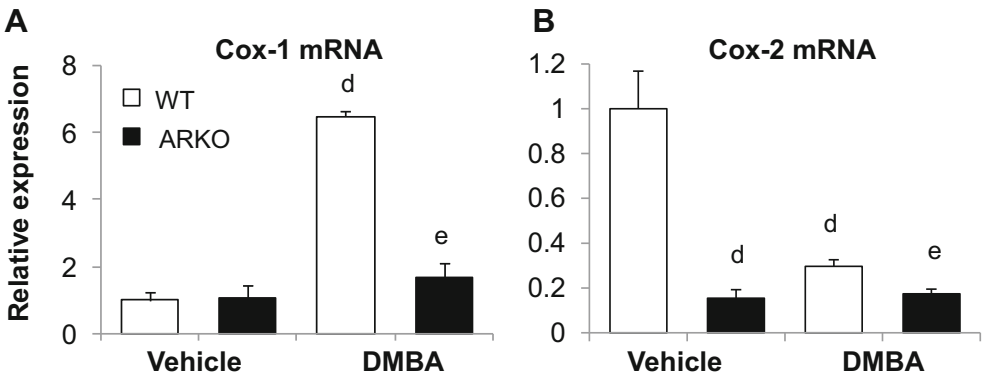

Fig. 5 Relative expression of *Cox-1* and *Cox-2* mRNA in the skin of WT and ARKO male mice 3 days following exposure to a single systemic dose of 1 mg DMBA (in 100 μl sesame oil vehicle). Expression is normalized to two reference genes, *Rpl19* and *Cyclophilin*; their geometric mean and suitability were obtained using geNorm software analysis and expressed relative to vehicle-treated WT male. The *Cox-1* mRNA expression was significantly ($p<0.05$; two-way ANOVA) affected by the local DMBA treatment, and the effect was dependent on genotype ($p<0.05$; two-way ANOVA) as it was only induced in WT male skin but not in ARKO following DMBA exposure. Similarly, the mRNA expression for *Cox-2* was dependent on genotype and treatment ($p<0.001$; two-way ANOVA). *d*, significantly different from vehicle treatment; *e*, significantly different from DMBA-treated WT. Data shown as mean±SE; $N=8$ and 6 for vehicle-treated WT and ARKO and 10 and 7 for DMBA-treated WT and ARKO

in female mice suggesting higher susceptibility for females. This might be related to the other hormone-dependent disorders of the sebaceous gland like acne that is significantly higher among women than men [6]. While only detected in females, the sebaceous hyperplasias were rare and detected in both WT and ARKO females, suggesting that while gender may influence on the incidence, the androgen status in females did not have an effect on the histopathology of DMBA-induced papillomas.

The skin structure is dependent on gender in both mice and humans [2, 9]. AR inactivation reduced epidermal thickness in both males and females. While there was also a nonsignificant trend for a reduced epidermal thickness in females compared to males, the difference was small (about 10 % thinner in females). This is more similar to the two-stage carcinogenesis model where papilloma numbers were dependent on AR status but not gender. Environmental factors causing DNA damage giving rise to skin cancers occur predominately in the basal epidermis that contains dividing keratinocytes [11]. Therefore, it could be hypothesized that the thinner epidermis in androgen-nonresponsive mice exposes the basal layer for the local DMBA treatment, increasing susceptibility to papillomas.

Dermal thickness on the other hand was significantly greater in males compared to females and also thicker in WT mice compared to ARKO mice. The role of androgens in dermal thickness in males is also supported by previous experimental studies demonstrating the role of AR and androgens regulating skin dermal thickness [2]. However, this is the first study to demonstrate that the skin dermal thickness in females is also regulated by androgens acting via AR. Since there is also a decreased thickness of the dermis in ARKO females compared to ARKO males, the gender difference in dermal thickness could also be dependent on ovarian hormones as estrogen receptors (ER)α and ERβ are expressed in dermal skin fibroblasts [10] and estrogens are known to modify skin thickness [5]. In contrast to epidermal thickness, the trend observed in dermal thickness seemed to be similar to the effect of gender and AR inactivation on the average papilloma number in the systemic DMBA-induced skin carcinogenesis model. Comparable to dermis thickness, the thickness of collage layer in the skin was modified by both AR inactivation and gender. This is supported by a previous study where androgen-insensitive males were shown to have reduced collagen content (measured as hydroxyproline content) in the dermal biopsy [16] as the hydroxyproline content would correlate with the thickness of the collagen layer. These findings suggest that either the skin structure is influencing DMBA-induced skin cancer susceptibility in mice or the same unknown factor(s) could be modifying the dermal/epidermal thickness and cancer susceptibility following DMBA treatment.

In conclusion, we have demonstrated that both gender and the androgens acting via AR significantly influence experimental, DMBA-induced skin cancer incidence. The effect of AR inactivation appeared to be dependent on the method of skin cancer induction (systemic DMBA vs. DMBA/TPA local, two-stage carcinogenesis). This could be due to different carcinogen mechanisms between the models and can be used in the future to further dissect the mechanisms of androgen and gender effects on skin cancer susceptibility.

Conflict of Interest Authors declare no conflict of interest.

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