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Modulation of basal and squamous cell carcinoma by endogenous estrogen in mouse models of skin cancer

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Patched1 **heterozygous mice (*Ptch1*$^{+/-}$) are useful for basal cell carcinoma (BCC) studies, being remarkably susceptible to BCC induction by ultraviolet or ionizing radiation. Analogously, skin carcinogenesis-susceptible (Car-S) mice are elective for studies of papilloma and squamous cell carcinoma (SCC) induction. We previously reported a striking effect of gender on BCC induction in *Ptch1*$^{+/-}$ mice, with total resistance of females; likewise, Car-S females show increased skin tumor resistance relative to males. Here, we investigated the protective role of endogenous estrogen in skin keratinocyte tumorigenesis. Control (CN) and ovariectomized *Ptch1*$^{+/-}$ or Car-S females were irradiated for BCC induction or topically treated with chemical carcinogens for SCC induction. Susceptibility to BCC or SCC was dramatically increased in ovariectomized *Ptch1*$^{+/-}$ and Car-S females and restored to levels observed in males. Remarkably, progression of initially benign papillomas to malignant SCC occurred only in ovariectomized Car-S females. We explored the mechanisms underlying tumor progression and report overexpression of estrogen receptor (ER)-α, downregulation of ERβ and upregulation of cyclin D1 in papillomas from ovariectomized Car-S relative to papillomas from CN females. Thus, an imbalanced ERα/ERβ expression may be associated with estrogen-mediated modulation of non-melanoma skin carcinogenesis, with a key role played by cyclin D1. Our findings underscore a highly protective role of endogenous estrogen against skin tumorigenesis by diverse agents in two independent mouse models of skin cancer.**

Introduction

The incidence of skin cancer has shown a rapid increase over the past few decades with >1 million new cases of non-melanoma skin cancer (NMSC) each year in the US (1). While ultraviolet (UV) radiation is the principal etiologic factor (2,3), ionizing radiation is also an established cause of human NMSC (4,5). Male sex is a known risk factor (6–8), and this gender influence has been mostly attributed to occupational exposures to UV radiation (9) and less use of sun-protective measures in men compared with women (10,11). However, there is a paucity of epidemiological data on the incidence of NMSC in males and females of equivalent sociologic context and with similar lifestyle. NMSC include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), representing the most common human cancers in many countries (12).

BCC, that makes up 75–80% of NMSC, is the most frequent cancer in whites and its incidence is increasing worldwide (13). The principal etiologic factors in human BCC include UV and ionizing radiation and chemical carcinogens (9). At cellular and molecular level, the importance of deregulation of the Sonic Hedgehog (SHH) pathway in epidermal keratinocytes as a primary event in the pathogenesis of BCC is well established (14–18). The SHH receptor, patched (PTCH), represses SHH signaling by inhibiting the signaling effector smoothened. PTCH mutational inactivation leads to overexpression of downstream SHH-responsive genes, such as *GLI1* and *GLI2*, which results in BCC development (19,20). Mice heterozygous for mutations in *Ptch1* (*Ptch1*$^{+/-}$) (21,22) develop BCC-like tumors following UV or ionizing radiation (22–24). We have previously identified a striking effect of gender on BCC induction by ionizing radiation in *Ptch1*$^{+/-}$ mice (23). Macroscopically detectable, infiltrative BCCs, in fact, were induced only in males, and this strong gender bias was not influenced by genetic background in subsequent investigations of radiogenic BCC (25).

SCC is the second most common skin cancer, comprising approximately one-fifth of all cases of NMSC, with marked geographic variability (13). As BCC, this is a most common tumor in elderly patients, and it is usually the result of a high lifetime cumulative dose of solar radiation. However, ionizing radiation, chemicals and human papilloma virus infection may also lead to SCC (26). Early studies of chemically induced mouse skin carcinogenesis showed a high frequency of a codon 61 mutation of the c-Ha-*ras* gene in papillomas initiated with 9,10-dimethyl-1,2-benzanthracene (DMBA) and promoted with the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (27). Previously, we produced the carcinogenesis-susceptible (Car-S) outbred mouse line by selective breeding for high sensitivity to two-stage chemical carcinogenesis continued over several generations (28). Car-S mice are extremely susceptible to chemical induction of papillomas and SCCs. Throughout the generations of selection of Car-S mice, induction of papillomas and SCCs was prevalent in males compared with females, and male mice had shorter tumor latency (A.Saran, unpublished data). Additionally, it has been reported that male Skh-1 mice are more susceptible than females to induction of papillomas and SCCs by chronic UVB exposure (29); this result was attributed to differential oxidative DNA damage and antioxidant capacities in the skin depending on sex. These and previous data from studies in rodents (30) suggest that estrogens may be key modulators of skin tumorigenesis.

The skin is an important estrogen-responsive tissue, and the fundamental role of estrogen in the regulation of hair follicle cycling and self-renewing is well known (31). Estrogens inhibit hair growth by delaying the initiation of anagen (i.e. phase of follicle growth) and lengthening the duration of telogen (i.e. resting phase after follicle degeneration in catagen). This was shown in early studies with ovariectomized rats (32) and by more recent work assessing the direct effect of estrogens and antiestrogens in mouse skin (33–35). The effects of estrogens are mediated by estrogen receptor (ER)-α and ERβ, members of the nuclear steroid receptor superfamily. Both ERs have been detected in the skin of rodents and humans, though with distinct expression patterns (36); specifically, ERβ has been indicated as the predominant ER in human scalp skin (37), whereas in murine skin both ERs are expressed during hair follicle cycling in hair cycle-dependent manner (38).

We have been interested in exploring the relative contribution of estrogens to the sexual dimorphism of skin tumorigenesis. We therefore investigated the consequences of decreased estrogen levels due to ovariectomy on epithelial tumorigenesis by radiation or chemicals in

Abbreviations: BCC, basal cell carcinoma; CN, control; Car-S, carcinogenesis susceptible; DMBA, 9,10-dimethyl-1,2-benzanthracene; ER, estrogen receptor; NMSC, non-melanoma skin cancer; OVX, ovariectomized; PTCH, patched; SCC, squamous cell carcinoma; SHH, Sonic Hedgehog; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; UV, ultraviolet.

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well-characterized mouse models of skin cancer. In ovariectomized *Ptch1*$^{+/-}$ and Car-S females, basal and squamous tumor induction were drastically increased over intact controls (CNs), and restored to levels observed in males, showing that endogenous estrogens play a critical role in protection against BCC and SCC carcinogenesis by diverse agents in mouse skin. In addition, analysis of skin papillomas from ovariectomized and intact Car-S females suggests a role for the opposing action of ERs on cyclin D1 gene expression in modulation of squamous cell tumor induction and malignant progression.

Materials and methods

Mice

Mice lacking one *Ptch1* allele (*Ptch1*$^{neo67/+}$, named *Ptch1*$^{+/-}$ throughout the text), derived by gene targeting of 129/Sv embryonic stem cells and maintained on outbred CD1 background (21), were housed in the animal facility at ENEA-Casaccia (Rome, Italy) and genotyped as described (23). For producing the Car-S line, a highly genetically polymorph foundation population (F0) was obtained by balanced intercrossing of eight inbred mouse strains (A, DBA2, P, SWR, SJL, CBA, BALB/c and C57Bl/6). The two-stage initiation/promotion skin carcinogenesis protocol was used (single application of DMBA and repeated application of TPA twice weekly for promotion). Selective breeding for 19 consecutive generations was carried out based on the individual number of tumors at the end of the promotion period, with parents of consecutive generations selected among mice presenting the largest number of tumors (28).

Female *Ptch1*$^{+/-}$ ($n = 51$) and Car-S mice ($n = 27$) were anesthetized (65 mg/kg sodium pentobarbital intraperitoneally) and ovariectomized at 30 days of age [ovariectomized (OVX) groups]. At necropsy, the success of ovariectomy was checked by marked atrophy of the uterine horns. Thirty-six *Ptch1*$^{+/-}$ and thirty-four Car-S females were left intact as CN groups. Experimental protocols were reviewed by the Institutional Animal Care and Use Committee.

BCC induction by radiation

Irradiation of *Ptch1*$^{+/-}$ mice was performed using a Gilardoni CHF 320G X-ray generator (Gilardoni S.p.A., Mandello del Lario, Lecco, Italy; half value layer = 1.6 mm Cu) operated at 250 kVp, 15 mA, with filters of 2.0 mm Al and 0.5 mm Cu. OVX and CN *Ptch1*$^{+/-}$ females were whole-body irradiated (3 Gy of X-rays) at the estimated age of the second telogen of hair cycle, i.e. 60 days (CN, $n = 25$; OVX, $n = 24$), or of the third anagen, i.e. 90 days (CN, $n = 11$; OVX, $n = 27$). Mice were observed daily for their whole life span; at the first signs of morbidity, they were killed and complete autopsies were performed. Tumor parameters were expressed as the percentage of mice bearing one or more macroscopic BCC-like tumors and the mean number of tumors per positive mouse in each group.

Induction of squamous skin tumors by two-stage carcinogenesis

Groups of 60- and 90-day-old OVX and CN Car-S females were initiated with a single DMBA application (1 μg/100 μl acetone; Sigma–Aldrich Co, St Louis, MO) on shaven dorsal skin. Promotion started 7 days after initiation and continued with 2 weekly TPA applications (0.25 μg/100 μl acetone; Sigma–Aldrich Co.). Twice weekly, papillomas with diameter ≥1 mm were recorded by investigators blinded to treatment groups. Tumor parameters were expressed as the percentage of mice bearing one or more papillomas (tumor incidence) and the average number of papillomas in the total number of mice per group, including non-tumor-bearing animals (tumor multiplicity ± standard error). Tumor latency was expressed as the number of days of promotion preceding appearance of the first tumor, calculated from slope extrapolation on the *x*-axis. Tumor induction rate was expressed as the mean tumor multiplicity at the end of promotion divided by the number of promotion days (expressed per 10 day periods). Four months after promotion end, mice were inspected for recording of papilloma progression to carcinoma. Malignant conversion is the ratio between mean of carcinoma and papilloma multiplicity.

Histological analysis

Normal skin of OVX and CN *Ptch1*$^{+/-}$ and Car-S mice of 45, 60, 75, 90 and 120 days was processed for histological analysis using standard methods. Skin samples from OVX and CN mice of 60 and 90 days were snap frozen and stored at −80°C. Skin tumors from *Ptch1*$^{+/-}$ and Car-S mice were also processed for histology or stored at −80°C for immunoblot analysis.

Immunohistochemistry

Immunohistochemical analysis of polyclonal antibody against Ki67 (Novocastra, Novocastra Laboratories, Newcastle, UK) was carried out on 3 μm thick paraffin sections as described (24). Immunohistochemical scoring was carried out by investigators blinded to treatment groups. The number of positive (brown stained) cells was determined as the percentage of the total number of cells counted in 0.5 mm of basal lamina in six different papillomas per study group (i.e. CN and OVX Car-S).

RNA extraction and reverse transcription–polymerase chain reaction

Total RNA was isolated from normal skin of OVX and CN *Ptch1*$^{+/-}$ mice of 60 and 90 days using NucleoSpin Extract (Macherey-Nagel, Duren, Germany) and stored at −80°C until further processing. Total RNA (2 μg) was reverse transcribed and amplified for *Gli1*, as described (24).

Immunoblotting

Samples (50 μg aliquots as determined by the Bradford assay; Bio-Rad Laboratories, Hercules, CA) were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes Hybond P+ (Amersham Biosciences, Buckinghamshire, UK). Blots were developed using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Proteins were visualized by chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL). Protein levels were quantified by densitometric analysis using the Scion Image Beta 4.02 software package (Scion Corporation, Frederick, MD). Filters were stripped and reprobed with anti-β-actin antibody for normalization. Antibodies used include rabbit polyclonal antibody against ERα (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody against ERβ (1:500 dilution; Upstate, Charlottesville, VA) and mouse monoclonal antibody against cyclin D1 (1:1000 dilution; Santa Cruz Biotechnology). Immunoblotting was performed on papillomas from OVX and CN Car-S mice (i.e. three tumors from each group and initiation time). Similar analyses of BCCs from *Ptch1*$^{+/-}$ or SCCs from Car-S mice were not feasible, due to scant tumor induction in CN mice.

Statistics

Analyses were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA). We used Mann–Whitney test for comparison of tumor multiplicity and Fisher's exact test for analysis of tumor incidence. *P* values are for two-sided tests; *P* values < 0.05 were considered statistically significant.

Results

Ovariectomy restored BCC responsiveness in Ptch1$^{+/-}$ *females*

To assess the role of estrogen deprivation on BCC development, OVX and CN *Ptch1*$^{+/-}$ mice irradiated with 3 Gy of X-rays at 60 or 90 days (i.e. the estimated ages of the second telogen and third anagen of hair cycle) were monitored throughout the life span for development of macroscopic BCC.

Macroscopically, BCCs developing in *Ptch1*$^{+/-}$ mice appeared as translucent scarlet papules often superficially ulcerated. Histological features showed dense tumor nodules with margins characterized by cylindrical tumor cell chains arranged in a palisade pattern, resembling infiltrating human BCC (Figure 1A).

The kinetics of tumor induction in mouse groups is shown in Figure 1B. Final BCC incidences were 25% (6/24) and 14.8% (4/27) in OVX-60 and OVX-90 *Ptch1*$^{+/-}$ mice, respectively. When compared with incidences observed in irradiated CN *Ptch1*$^{+/-}$ mice (i.e. 4% in CN-60 *Ptch1*$^{+/-}$ and 0% in CN-90 *Ptch1*$^{+/-}$), these values strongly suggest an influence of hormone status on BCC development. Overall, 19.6% of OVX *Ptch1*$^{+/-}$ mice (10/51) developed macroscopic BCCs, compared with 2.8% (1/36) in CN *Ptch1*$^{+/-}$ mice, a 7-fold difference ($P = 0.02$). Notably, in OVX *Ptch1*$^{+/-}$ mice, susceptibility to BCC was re-established to similar or higher levels than previously observed in males, i.e. 13 and 14% after irradiation at 60 or 90 days, respectively [(23,24) and data not shown].

OVX *Ptch1*$^{+/-}$ mice showed a mean tumor latency of 50 weeks. Comparisons of latency with CN *Ptch1*$^{+/-}$ females are not feasible since only one of 36 females with intact ovarian function—monitored for over 120 weeks—developed a BCC (i.e. at 38 weeks from irradiation at 60 days). However, it is remarkable that OVX latencies were very similar to those previously observed in similarly irradiated intact males (i.e. 45 weeks) (23,24).

Multiple BCCs developed in OVX *Ptch1*$^{+/-}$ mice irradiated at 60 days, with 2.5 ± 1.0 tumors per positive mouse. In OVX *Ptch1*$^{+/-}$ mice exposed at 90 days, tumor multiplicity was 1.25 ± 0.3. Again, due to almost total lack of BCC induction in females, the multiplicity parameter can only be compared with that previously observed in

intact *Ptch1*$^{+/-}$ males, in which macroscopic BCCs developed always as solitary tumors (23,24).

To investigate whether ovariectomy could contribute to deregulation of Shh/Ptch1 signaling in normal unirradiated *Ptch1*$^{+/-}$ skin, we quantified by reverse transcription–polymerase chain reaction *Gli1* messenger RNA levels in normal skin of CN and OVX *Ptch1*$^{+/-}$ mice. No significant differences in *Gli1* expression were detected between normal skin from CN and OVX *Ptch1*$^{+/-}$ mice at either ages (60 and 90 days; Figure 1C and D), suggesting no major interference of estrogen deprivation with Shh/Ptch1 signaling.

Ovariectomy increased response to two-stage carcinogenesis in Car-S females

We have long observed that in Car-S mice, which we phenotypically selected for susceptibility to two-stage skin carcinogenesis by a long-term breeding program (28), females showed resistance to papilloma and SCC induction by DMBA/TPA relative to males (A.Saran, unpublished results). This observation was confirmed by using direct-acting initiators, i.e. *N*-methyl-*N*-nitrosourea (supplementary Table S1 is available at *Carcinogenesis* Online) (39). To test the hypothesis that estrogens exert protective effects on squamous cell papillomas and carcinomas, in addition to BCC, we subjected OVX and CN Car-S females to a classical two-stage protocol; papilloma incidence, multiplicity, induction rate and progression to malignant carcinoma were examined. At promotion end, all tumor parameters were higher in OVX mice compared with CN groups (Figure 2 and Table I). In mice initiated at 60 days, tumor incidence reached 100% after 53 and 67 promotion days in OVX and CN females, respectively (Figure 2A). In mice initiated at 90 days, only the OVX group reached 100% tumor incidence after 70 promotion days, whereas CN mice attained a plateau incidence of 86% after 95 days (Figure 2D). Differences in tumor multiplicity were statistically significant in both initiation groups, with a multiplicity of 13.5 ± 2.1 in OVX versus 7.9 ± 0.9 in CN-60 ($P = 0.0167$) and a multiplicity of 18.4 ± 1.6 in OVX versus 10.9 ± 1.9 in CN-90 mice ($P = 0.0071$; Figure 2B and E and Table I).

Tumors developing in Car-S mice during TPA promotion had macroscopic aspect of papillomas characterized by outward growth (Figure 2G), and their benign nature was confirmed by sporadic histology. Some of the papillomas progressed to SCCs, showing characteristic downward growth, with dermis and subcutaneous infiltration (Figure 2H). Their malignant nature was always confirmed by histology at the end of experiment. The most prominent effect of ovariectomy was a drastic increase in SCC, with 45 and 38% incidence in OVX versus 0 and 7% in CN mice after initiation at 60 or 90 days, respectively (Figure 2C and F and Table I). Differences were statistically significant between OVX-60 and CN-60 groups ($P = 0.0441$) and approached statistical significance in OVX-90 and CN-90 groups ($P = 0.08$).

Effects of ovariectomy on hair cycling

Because skin tumorigenesis is influenced by hair growth cycling (24,40), with higher susceptibility in anagen, we analyzed the effects of ovariectomy on synchronous hair cycling in young mice. For evaluation of estrogen deprivation effects on hair growth, we conducted histological analysis of dorsal skin sections from CN and OVX *Ptch1*$^{+/-}$ and Car-S mice at various ages (Figure 3). In both strains, there was prominent hair cycle perturbation in the skin of ovariectomized mice at 45 and 60 days, that showed the typical architecture of anagen phase, with maximal extension of hair follicles and location of dermal papillae in the deep subcutis (i.e. anagen V–VI) (41). The age-matching skin of CN mice, in contrast, was in telogen, with follicles residing entirely in the dermis, absence of inner root sheath and round, compact, quiescent dermal papillae. Thus, ovariectomy at 30 days (i.e. beginning of second anagen) caused hair follicles to remain in anagen at times normally corresponding to early and middle part of the second synchronized telogen. At 75 days, however, the skin of OVX mice was in telogen, similar to skin of CN mice. At 90 days, the skin of OVX and CN mice showed again similar architecture, with features of nearly mature anagen (i.e. anagen IIIc–IV) (41). No hair cycle perturbation was observed at later times, i.e. in telogen skin at 120 days. In addition, no differences in hair cycling, or in hair cycle perturbation by ovariectomy, were detected between *Ptch1*$^{+/-}$ and Car-S mice.

Expression of ERα and ERβ in normal skin and papillomas from Car-S mice

The biological responses to estrogens at tissue level are mediated by ERs. By immunoblot analysis, we have determined ERs level in

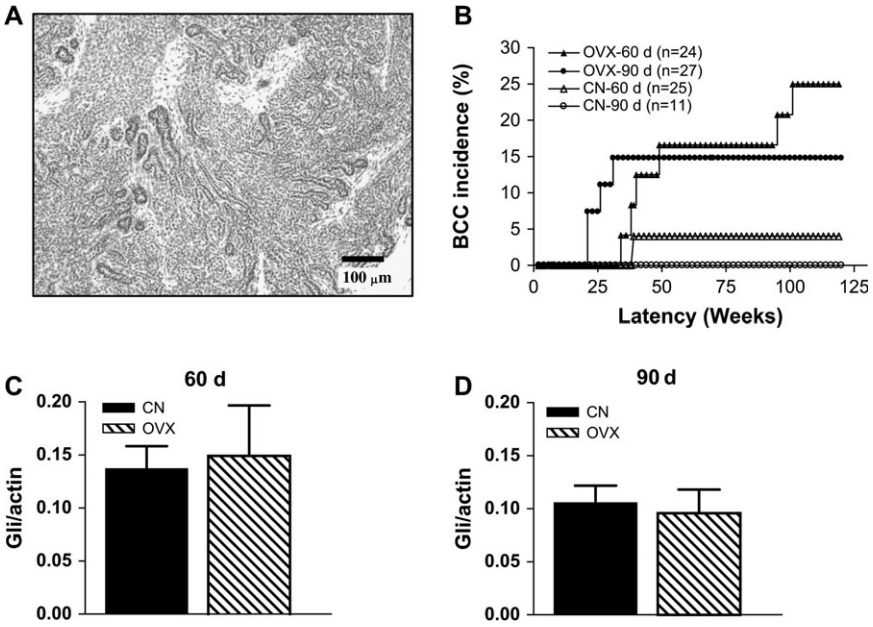

Fig. 1. Histology and incidence of BCC-like tumor in irradiated *Ptc1*$^{+/-}$ female mice. (**A**) Typical BCC-like tumor characterized by the presence of irregular basaloid cell nests with typical peripheral cell palisading and lack of follicular differentiation, resembling human BCC. (**B**) Percent BCC incidence in OVX and CN *Ptc1*$^{+/-}$ mice irradiated at 60 or 90 days. OVX-60 *Ptc1*$^{+/-}$ females had a 25% BCC incidence, compared with 4% in CN-60 mice, a 6.3-fold difference ($P = 0.049$). Irradiation at 90 days induced BCCs (15%) only in OVX *Ptc1*$^{+/-}$ mice. (**C** and **D**) Normalized expression levels of *Gli1* in normal unirradiated skin of CN and OVX *Ptc1*$^{+/-}$ mice of 60 and 90 days. Three independent polymerase chain reaction runs were carried out for each mouse, and β-actin was used as a reference standard for all analyses. Data represent the ratios of final averages for each group ($n = 4$). Bars, standard error.

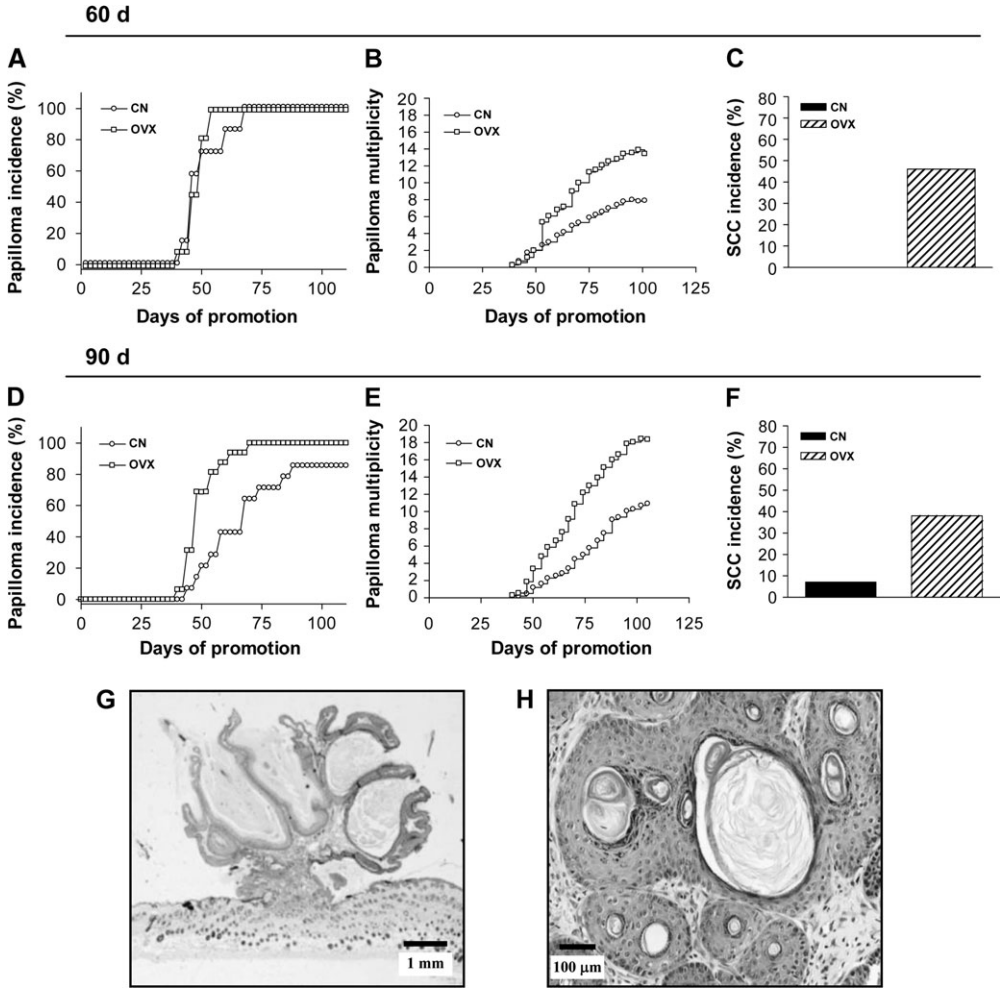

Fig. 2. Papilloma and SCC induction after two-stage carcinogenesis. (**A** and **D**) Percent incidence and (**B** and **E**) tumor multiplicity in groups of OVX and CN Car-S females initiated at 60 or 90 days with DMBA (1 µg) and promoted twice a week with TPA (0.25 µg). (**C** and **F**) SCC incidence detected in Car-S mice 4 months after the end of promotion. Differences were statistically significant between OVX and CN groups initiated at 60 days (C; $P = 0.0441$) and approached statistical significance for groups initiated at 90 days (F; $P = 0.08$). (**G**) Histology of large regular papilloma with typical cauliflower-like structure consisting of a series of folds united by a stalk to the underlying skin. (**H**) Histology of a well-differentiated SCC invading the dermis; note the presence of large areas filled with horny material.

Table I. Skin tumorigenesis induced by DMBA initiation (1 µg) and twice weekly TPA promotion (0.25 µg) in Car-S mice

Group	Number of mice	Promotion (days)	Papillomas at the end of promotion				Carcinomas 220 days after promotion start		
			Tumor latency (days)[a]	Incidence (%)	Multiplicity ($\bar{X}$±SE)	TIR per 10 days ($\bar{X}$±SE)	Mice with tumors (%)	Multiplicity ($\bar{X}$±SE)	Malignant conversion (%)
CN-60	20	101	32	100	7.90 ± 0.86	0.78 ± 0.09	0 (0)	0	0
OVX-60	11	101	34	100	13.45 ± 2.12[b]	1.33 ± 0.43	5 (45)[c]	0.70 ± 0.25	5
CN-90	14	105	44	86	10.93 ± 1.88	1.04 ± 0.18	1 (7)	0.07 ± 0.07	0.6
OVX-90	16	105	38	100	18.38 ± 1.60[d]	1.75 ± 0.15	6 (38)[e]	0.44 ± 0.16	2

TIR = tumor induction rate, SE = standard error.
[a]Tumor latency was expressed as the number of days of promotion preceding the appearance of the first tumor, calculated from slope extrapolation on the *x*-axis.
[b]$P = 0.0167$.
[c]$P = 0.0441$.
[d]$P = 0.0071$.
[e]$P = 0.08$.

untreated skin samples from CN and OVX Car-S mice at day 60 and 90 (supplementary Figure 1 is available at *Carcinogenesis* Online). Results showed that in normal skin from Car-S females, ERα expression is hair cycle dependent, with maximal expression in telogen skin (i.e. intact females at 60 days of age) and significantly reduced expression in late anagen phase (i.e. ovariectomized females at 60 days). At 90 days of age, there were no differences in ERα expression between the experimental groups, which were in the same phase of hair cycle (medium anagen). No significant changes were observed in ERβ expression, regardless of age and hair cycle phase.

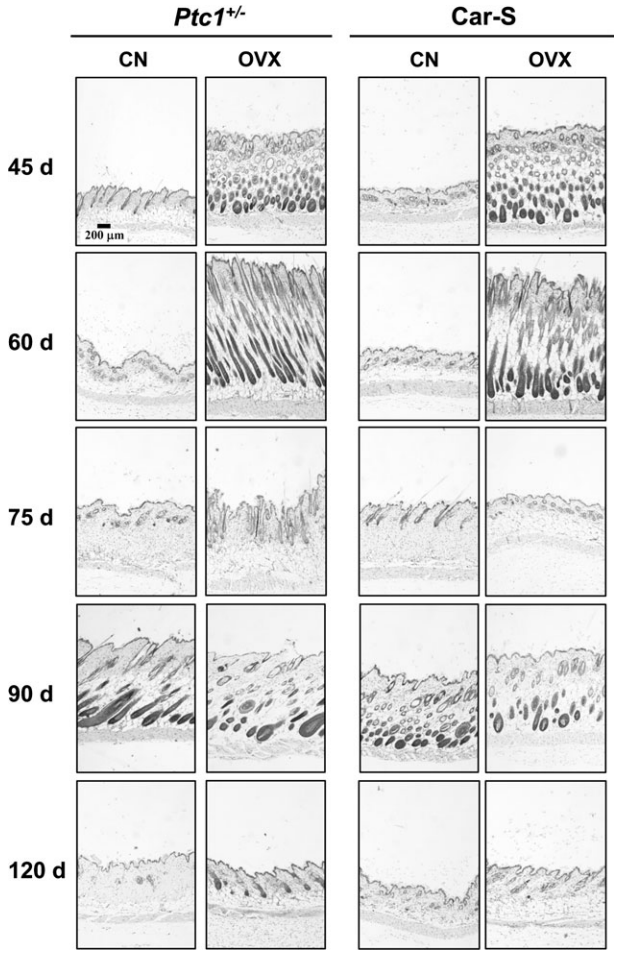

Fig. 3. Histological analysis of dorsal skin sections from CN and OVX *Ptc1*$^{+/-}$ and Car-S mice. At 45, 60 and 75 days of age, the skin of CN mice showed the typical architecture of a quiescent phase (telogen) of hair cycle, with hair follicles fully enclosed by the dermis; in contrast, at 45 and 60 days, the skin of ovariectomized mice showed features characteristic of mature anagen, characterized by maximal extension of hair follicles. At 75 days, hair follicle cycling was re-established in OVX females, and skin architecture was similar to that of CN females; no additional perturbation was observed at later times (i.e. 90 and 120 days).

The relative expression of ERs may shift with malignant progression. Malignant tumors (i.e. BCC and SCC) cannot be analyzed in our models, due to virtually complete lack of induction in intact females. Therefore, to gather information on the possible mechanisms underlying protective effects by estrogens, we analyzed expression of ERs in papillomas from OVX and intact Car-S females. Since very similar protein levels were observed in tumors from the 60 and 90 days group in OVX or CN females, data from the two time points were pooled. By immunoblot, we detected increased expression of ERα and downregulation of ERβ in tumors from OVX compared with tumors from CN mice (Figure 4A–C). Differences in expression levels were statistically significant for both ERα ($P = 0.0151$) and ERβ ($P = 0.0469$). The ratio ERα:ERβ was 0.13 in tumors from CN and 1.60 in tumors from OVX mice, a 12-fold difference (Figure 4D). This suggests a role of the ratio ERα:ERβ in susceptibility of the skin to estrogen-modulated carcinogenesis.

Expression of cyclin D1 and Ki67 in papillomas from Car-S mice

Because a complex relationship between ERs and cyclin D1 has been established (42,43), we examined cyclin D1 protein levels in tumors from OVX and CN mice (Figure 5A). Significantly, we observed a remarkably increased expression of cyclin D1 in OVX relative to CN tumors ($P < 0.0001$).

Immunohistochemistry was carried out on tumor sections using an antibody directed against the proliferation marker Ki67. The percentage of Ki67-positive cells was highly increased in papillomas from OVX compared with CN females (Figure 5B); the mean values of positively staining cells per papilloma were 71.1 ± 3.7 and 43.9 ± 4.0% in the OVX and CN groups, respectively ($P = 0.0003$), showing significantly higher proliferative potential of papillomas from OVX mice.

Discussion

Although it is clear that estrogens may stimulate tumor development in some hormone-responsive tissues such as the breast and the uterus (44,45), their role in other malignancies remains actually unclear. Thus, if on one hand estrogen has been classified as carcinogenic to humans by the International Agency for Research on Cancer (46), on the other hand various experimental studies suggest that it may exert a protective role against development of other malignancies, such as colon cancer (47). Accordingly, a reduced relative risk and mortality from this pathology have been reported in women receiving hormone replacement therapy (48). Besides the classical steroid-hormone susceptible and -producing organs, 17-β-estradiol is also produced by many non-ovarian tissues (e.g. adipose tissue, brain, testes, etc.) and it can exert profound effects in non-reproductive organs in both females and males (49). The skin locally synthesizes significant amounts of sexual hormones with intracrine or paracrine actions. However, the local level of each sexual steroid depends on the expression of androgen- and estrogen-synthesizing enzymes in different cell types (50). The role of estrogen in the regulation of hair follicle cycling in mice was rediscovered in the past decade, following a seminal paper by Oh *et al.* (33), showing that an ER pathway within the dermal papilla regulates the telogen–anagen follicle transition and that 17-β-estradiol blocks hair growth and arrests hair follicles in telogen.

Experimental data from the present study support the concept that female sex hormones can be protective in non-melanoma skin carcinogenesis; in fact, we found that skin tumor development was significantly enhanced after ovarian hormone withdrawal in two independent experimental models. The results shown here demonstrate increased skin tumor incidence and multiplicity and decreased tumor latency in ovariectomized versus CN females, regardless of the nature of the keratinocyte-initiating agent (i.e. chemical for SCC and physical for BCC). Remarkably, malignant progression of benign papillomas to SCC occurred almost exclusively in OVX Car-S and was rare in CN females following two-stage carcinogenesis by DMBA/TPA.

BCC induction in mouse skin can be strongly affected by hair growth cycling (24). Similarly, hair cycle-dependent susceptibility to squamous cell papillomas developing after one- or two-stage chemical carcinogenesis has been reported earlier (40) and confirmed in Car-S mice (M.Mancuso, unpublished results). Here, we show that increased skin tumor susceptibility in ovariectomized mice was not a consequence of hair cycle alteration caused by estrogen withdrawal, as perturbations (i.e. follicles remaining or entering anagen in periods corresponding to a quiescent phase) were transitory in both strains, and observed only in mice of 45 and 60 days. There were no effects at 90 days (i.e. hair follicles were in anagen in OVX and CN groups). Nevertheless, ovariectomized *Ptch1*$^{+/-}$ and Car-S females of both groups were remarkably more susceptible to BCC and SCC induction compared with intact mice. Notably, hair cycle changes were no longer observed at intermediate and later times, i.e. 75 and 120 days. Thus, hair cycle perturbation by estrogen deprivation is transient and cannot represent the factor reversing skin tumor resistance in females. Moreover, we show that BCC susceptibility of *Ptch1*$^{+/-}$ skin is not due to further Shh/Ptch1 signaling deregulation in estrogen-deprived females, as by reverse transcription–polymerase chain reaction no overexpression of *Gli1* transcriptional target of Shh signaling was present in normal skin from OVX compared with CN *Ptch1*$^{+/-}$ mice at both times examined.

Carcinogen metabolism is known to be suppressed by estrogen (51) and might play a role in initiation by chemical agents. However, we

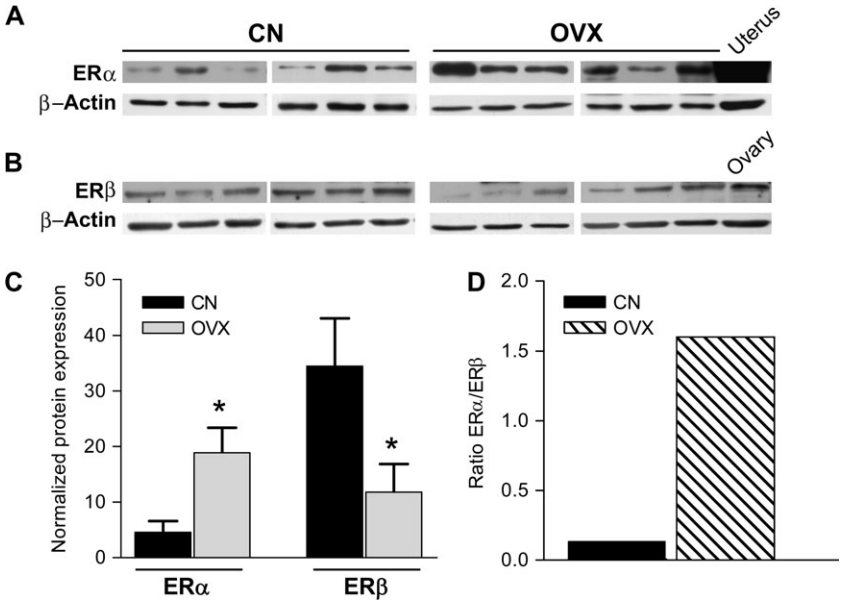

Fig. 4. ERα and ERβ are differentially expressed in tumors from ovariectomized and intact Car-S females. (**A** and **B**) Immunoblot analysis of ERα and ERβ in papillomas from OVX and CN Car-S mice. Protein extracts from uterus and ovary were used as positive CNs for ERα and ERβ, respectively. (**C**) Graphic representation of normalized ERα and ERβ protein levels in papillomas from OVX and CN mice. Columns represent the mean ± standard error of six tumors per each group. Differences were statistically significant between tumors from OVX and CN groups (ERα, $^{*}P = 0.0151$; ERβ, $^{*}P = 0.0469$). (**D**) Ratio of ERα to ERβ protein expression, based on densitometry results, is shown for OVX and CN groups.

have shown previously that activation or detoxification of polycyclic aromatic hydrocarbons is not a predominant factor in either Car-S responsiveness to chemical skin carcinogenesis (52) or in gender differences. Sex difference, in fact, was not specific of the DMBA/TPA protocol, as Car-S males and females tested for initiation with *N*-methyl-*N*-nitrosourea, a highly reactive compound not requiring metabolic activation for carcinogenic activity, showed highly significant differences in papilloma and SCC induction. This suggests that polycyclic hydrocarbon metabolism is not a prevalent factor in the dimorphism between intact and estrogen-deprived Car-S females. Because diverse tumor induction protocols were used in the two mouse models, we favor the hypothesis of a general tumor suppressor role of estrogen in skin.

We have been intrigued by the observation that malignant tumor growth, such as BCC development, was induced preponderantly in OVX *Ptc1*$^{+/-}$ females and by its virtually complete lack in CN females; likewise, progression of initially benign papillomas to malignant SCC was manifestly predominant in OVX Car-S mice.

The role of estrogen levels in altering ERα and/or ERβ expression has been shown in different target tissues, with effects varying among different tissues, cell types and experimental protocols (53–55). In normal Car-S mouse skin, ERα expression was hair cycle dependent with maximal expression in telogen hair cycle phase; in addition, no significant changes were observed in ERβ expression, regardless of age and hair cycle phase. These results are in keeping with previous observation by Ohnemus *et al.* (38).

To shed light on potential mechanisms involved in estrogen modulation of skin tumor progression, we examined ER protein levels in benign skin papillomas from the different Car-S groups. Immunoblots of papilloma extracts showed significantly increased expression of ERα and downregulation of ERβ in tumors from OVX relative to CN tumors, suggesting a role of the ratio ERα:ERβ in susceptibility of skin to estrogen-modulated carcinogenesis, and a correlation of decreased ERβ expression with increased malignant progression of initially benign papillomas in ovariectomized Car-S mice.

Previous studies have established a complex relationship between ERs and cyclin D1, with important implications for proliferation of estrogen-responsive tissues and deregulation of proliferation in cancer (42,43). To further explore this issue, we analyzed tumors for expression of cyclin D1, which among D-type cyclins controlling cell cycle regulation has been most directly implicated in oncogenesis. In the presence of estrogen, cyclin D1 is one important target gene through which estrogen-complexed ERα mediates its proliferative action, whereas estrogen-complexed ERβ represses cyclin D1 gene transcription and blocks ERα-mediated induction when both receptors are present (56). In the absence of estrogen, however, cyclin D1 is able to bind to and activate transcription mediated by ERα (42,43,57). Significantly, we detected cyclin D1 upregulation in tumors from OVX relative to CN mice. Thus, our results suggest that in tumors from intact mice, where the ratio ERα:ERβ is low, the protective role of ERβ may be privileged over the proliferation stimulus mediated by the α-isoform, whereas in tumors from ovariectomized animals, the inverted ERα:ERβ ratio may favor proliferation and malignant progression, possibly due to the oncogenic role of cyclin D1. This hypothesis is supported by the higher proliferation rate observed in papillomas from OVX compared with intact CN mice, a finding also observed in ER-positive breast cancer, where high cyclin D1 expression correlates with high Ki67 expression (58). We cannot exclude, however, that ovariectomy may modulate other factors involved in the regulation of skin development and functions, such as progesterone levels (59) and that this modulation may in turn influence tumor development.

In summary, our study shows for the first time a protective role of endogenous estrogen against basal and squamous skin tumorigenesis caused by physical or chemical agents in independent mouse models. We also show that this protective role is not related with regulation of hair cycling by estrogen or with hair cycle perturbation by estrogen withdrawal. Finally, our study suggests that reciprocal expression of ERα and ERβ may be associated with estrogen-mediated modulation of squamous epithelial carcinogenesis, with a key role played by cyclin D1. Our results are in agreement with findings from several epidemiologic studies in humans reporting gender differences in NMSC and support the need to investigate new therapeutic approaches in the treatment of these diseases. Further work will be needed to establish whether ERα antagonists, or ERβ agonists, might be a useful addition in treatment of NMSC.

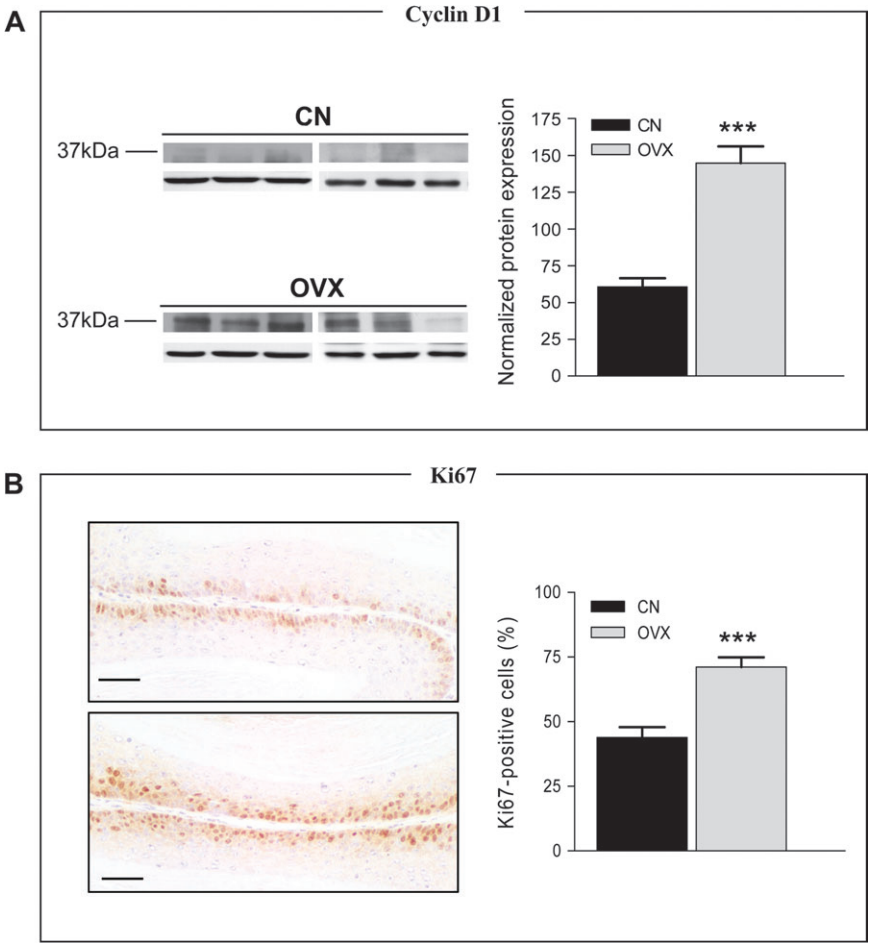

Fig. 5. Expression of cyclin D1 and Ki67 in tumors from ovariectomized and intact Car-S females. (**A**) Immunoblot analysis and graphic representation of normalized cyclin D1 levels in papillomas from OVX and CN Car-S mice. Columns represent the mean ± standard error of six tumors per each group. Differences were statistically significant between OVX and CN groups ($P < 0.0001$). (**B**) Representative Ki67 immunostaining and graphic representation of Ki67-positive cells (%) in papillomas from intact CN and OVX Car-S mice. Columns represent the mean ± standard error of six tumors per each group. Differences were statistically significant between OVX and CN groups ($P = 0.0003$). Bars, 50 μm.

Supplementary material

Supplementary Figure 1 and Table S1 can be found at http://carcin.oxfordjournals.org/

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