Malignant Transformation of DMBA/TPA-Induced Papillomas and Nevi in the Skin of Mice Selectively Lacking Retinoid-X-Receptor α in Epidermal Keratinocytes

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Retinoid-X-receptor α (RXRα), a member of the nuclear receptor (NR) superfamily, is a ligand-dependent transcriptional regulatory factor. It plays a crucial role in NR signalling through heterodimerization with some 15 NRs. We investigated the role of RXRα and its partners on mouse skin tumor formation and malignant progression upon topical DMBA/TPA treatment. In mutants selectively ablated for RXRα in keratinocytes, epidermal tumors increased in size and number, and frequently progressed to carcinomas. As keratinocyte-selective peroxisome proliferator-activated receptor gamma (PPARγ) ablation had similar effects, RXRα/PPARγ heterodimers most probably mediate epidermal tumor suppression. Keratinocyte-selective RXRα-null and vitamin-D-receptor null mice also exhibited more numerous dermal melanocytic growths (nevi) than control mice, but only nevi from RXRα mutant mice progressed to invasive human-melanoma-like tumors. Distinct RXRα-mediated molecular events appear therefore to be involved, in keratinocytes, in cell-autonomous suppression of epidermal tumorigenesis and malignant progression, and in non-cell-autonomous suppression of nevi formation and progression. Our study emphasizes the crucial role of keratinocytes in chemically induced epidermal and melanocytic tumorigenesis, and raises the possibility that they could play a similar role in UV-induced tumorigenesis, notably in nevi formation and progression to melanoma.

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INTRODUCTION

Retinoids (principally all-*trans* retinoic acid, the active vitamin A derivative) are involved in control (CT) of growth and differentiation of normal, premalignant, and malignant cell types (Altucci and Gronemeyer, 2001). Their effects are mediated through binding to members of two families of nuclear receptors (NRs), the retinoic acid (RA) (RARα, β, and γ), and retinoid X (RXRα, β, and γ) receptors, usually associated as heterodimers (Mangelsdorf *et al.*, 1995; Chambon, 1996; and reference therein). Vitamin A deficiency in humans and animal models results in epithelial squamous metaplasia prone to malignant conversion (Hong and Itri, 1994; Moon *et al.*, 1994), whereas natural and synthetic retinoids are used for treatment of skin disorders including psoriasis and acnea (Livrea, 2000), and as chemopreventive agents for epidermal cancers (Altucci and Gronemeyer, 2001; Sun and Lotan, 2002). Altered expression of RARs and RXRs during carcinogenesis may reduce the ability of epidermal cell to respond to RA, and contribute to carcinoma development, both in mice (Darwiche *et al.*, 1995; Darwiche *et al.*, 1996) and humans (Sun and Lotan, 2002). In mice, dietary RA prevents tumor formation and malignant conversion of chemically induced skin papillomas (De Luca *et al.*, 1996, and references therein; Tennenbaum *et al.*, 1998).

The two-step chemical tumorigenesis model has been widely used to study initiation, promotion, and progression

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Abbreviations: CT, control; DMBA, 7,12-dimethyl-benz[a]anthracene; FA, fluocinolone acetonide; MG, melanocytic growth; NR, nuclear receptor; PPAR, peroxisome proliferator-activated receptors; RXR, retinoid-X-receptor; SCC, squamous cell carcinoma; TAM, tamoxifen; VDR, vitamin-D-receptor

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(malignant conversion) of mouse skin tumors, as the evolution of human squamous cell carcinoma (SCC) has many similarities with mouse chemically induced squamous cell tumors (Yuspa, 1998, and references therein). The initiation stage is achieved through a single topical appplication of 7,12-dimethyl-benz[a]anthracene (DMBA). Promotion is accomplished by repeated topical application of a tumor promoter (i.e., phorbol ester 12-*O*-tetradecanoyl-phorbol-13 acetate (TPA)), which causes a sustained hyperplasia and inflammation, and then a selective clonal expansion into benign papillomas (Yuspa, 1998, and references therein). The third stage, progression, is the malignant conversion of benign papillomas to invasive SCC. Cutaneous papillomas and SCCs induced by DMBA/TPA treatment, as well as human SCC, have been shown to contain the Ha-ras gene codon 61 ($A^{182} \rightarrow T$) mutation (Nelson *et al.*, 1992, and references therein), and deletion of Ha-ras drastically decreases the incidence of DMBA/TPA-induced papillomas (Ise *et al.*, 2000). Interestingly, benign melanocytic dermal tumors (nevi) are induced in mouse skin upon a single topical DMBA application. However, these nevi in which N-ras, but not Ha-ras mutations at codon 61, have been found, never progress to malignant melanomas (Husain *et al.*, 1991).

RXRα, RXRβ, RARα, and RARγ are expressed in human and mouse epidermis, and RXRα and RARγ are the predominant receptors (Fisher and Voorhees, 1996; Chapellier *et al.*, 2002). The RXRα-null mutation is lethal *in utero* between gestation days 13.5 and 16.5 (Kastner *et al.*, 1994; Sucov *et al.*, 1994), precluding genetic studies of its physiological function in skin homeostasis. This became possible through conditional inactivation of RXRα using the Cre/loxP technology, and transgenic mice selectively expressing the tamoxifen (TAM)-inducible Cre-ERT2 recombinase in epidermal keratinocytes (Li *et al.*, 2000; Metzger *et al.*, 2003). RXRα ablation in keratinocytes of adult mice resulted in alopecia, epidermal keratinocyte hyperproliferation, and aberrant terminal differentiation, and skin inflammatory reaction (Li *et al.*, 2000, 2001). In addition to RARs, RXRs are also known to heterodimerize with other members of the NR superfamily (e.g., peroxisome proliferator-activated receptors (PPARs), vitamin-D-receptor (VDR), and liver-X-receptors (LXRs); (Laudet and Gronemeyer, 2002)) expressed in the epidermis (Li *et al.*, 2000; Fowler *et al.*, 2003; Mao-Qiang *et al.*, 2004; Schmuth *et al.*, 2004, and references therein; Sheu *et al.*, 2002). Ligands of PPARs and VDR have been shown to inhibit the growth of SCC cell lines *in vitro* and multistage skin tumorigenesis *in vivo* (Kensler *et al.*, 2000; Kopelovich *et al.*, 2002; Nikitakis *et al.*, 2002), and those of LXRs display anti-inflammatory activity against TPA-induced cutaneous inflammation (Fowler *et al.*, 2003). Thus, RXR(s) and in particular RXRα, could be critical in transducing cellular signals controlling skin carcinogenesis.

To investigate the possible role of RXRα in papilloma formation and their malignant conversion, as well as in melanocytic growth (MG) formation and progression to melanoma, we have subjected adult mice selectively ablated for RXRα in keratinocytes, to chemical two-step tumorigenesis. Upon DMBA/TPA treatment they exhibited an increased number of epidermal tumors, which were also bigger and progressed with a much higher frequency to malignant carcinoma, thus establishing a cell-autonomous role of RXRα in epidermal tumor suppression. These DMBA/TPA-treated mutants also developed MGs degenerating into invasive human melanoma-like MGs, which appear to be induced by paracrine signal(s) emanating from RXRα-ablated epidermal keratinocytes. We also investigated the role of several NRs as possible partners of RXRα in these tumor suppressions.

RESULTS

Keratinocyte-selective ablation of RXRα increases the formation and growth of epidermal tumors in DMBA/TPA-treated mice

$RXR\alpha^{ep-/-}$ (epidermal keratinocyte-selective $RXR\alpha^{L-/L-}$-null genotype) mice, as well as CT $RXR\alpha^{ep-/+}$ and $RXR\alpha^{L2/L2}$ mice carrying either one RXRα L^- null allele and one wild-type allele selectively in keratinocytes or two floxed RXRα L2 alleles, were generated by Tam treatment of K14-Cre-ER^{T2}/$RXR\alpha^{L2/L2}$, K14-Cre-ER^{T2}/$RXR\alpha^{L2/+}$, and $RXR\alpha^{L2/L2}$ littermates, respectively (Materials and Methods; Li *et al.*, 2000; Metzger *et al.*, 2003). All CT and $RXR\alpha^{ep-/-}$ mice were on a similar mixed genetic background (see Materials and Methods). CT and $RXR\alpha^{ep-/-}$ female mice were topically treated, 2 weeks after Tam administration, with DMBA, and then twice a week with TPA for 25–30 weeks ($RXR\alpha^{ep-/-(a)}$ mice, see Figure 1a, and Materials and Methods). After 7–8 weeks epidermal tumors developed in all mice. Their number and size increased with time (Figure 1b, and data not shown), but they were more numerous and bigger in $RXR\alpha^{ep-/-(a)}$ mice (Figure 1b–d, and data not shown). The number of tumors was 2-fold lower in CT males, but $RXR\alpha^{ep-/-(a)}$ males also exhibited twice as many tumors as CT littermates, and their size was also increased (not shown). All further work was performed on females. In all cases, DMBA initiation was required to generate these tumors, as none appeared with TPA alone (not shown).

To investigate whether RXRα was involved in tumor initiation and/or promotion, mice were DMBA-treated, and then with TPA, for 7 weeks before Tam-induced RXRα ablation ($RXR\alpha^{ep-/-(b)}$ mice, Figure 1a). Tumors appeared after 6–7 weeks of TPA treatment in CT and $RXR\alpha^{ep-/-(b)}$ mice, and by 30 weeks the latter had twice as many tumors as CT mice (Figure 1e and f, and data not shown). Thus, the lack of RXRα did not affect tumor initiation, whereas it enhanced tumor promotion. Accordingly, the H-ras codon 61 ''activating'' mutation (see Introduction) was similarly present in CT and $RXR\alpha^{ep-/-}$ tumors (not shown).

Epidermal tumors generated in $RXR\alpha^{ep-/-}$ mice have a ''high risk'' of malignant conversion

Changes in keratin expression during DMBA/TPA tumorigenesis are used to classify papillomas into ''high'' and ''low'' risk for malignant progression. Loss of keratins K1 and K10 expressed in suprabasal layers of normal epidermis and in ''low risk'' papillomas, and the concomitant appearance of K13, normally not expressed in epidermis, are indicators of

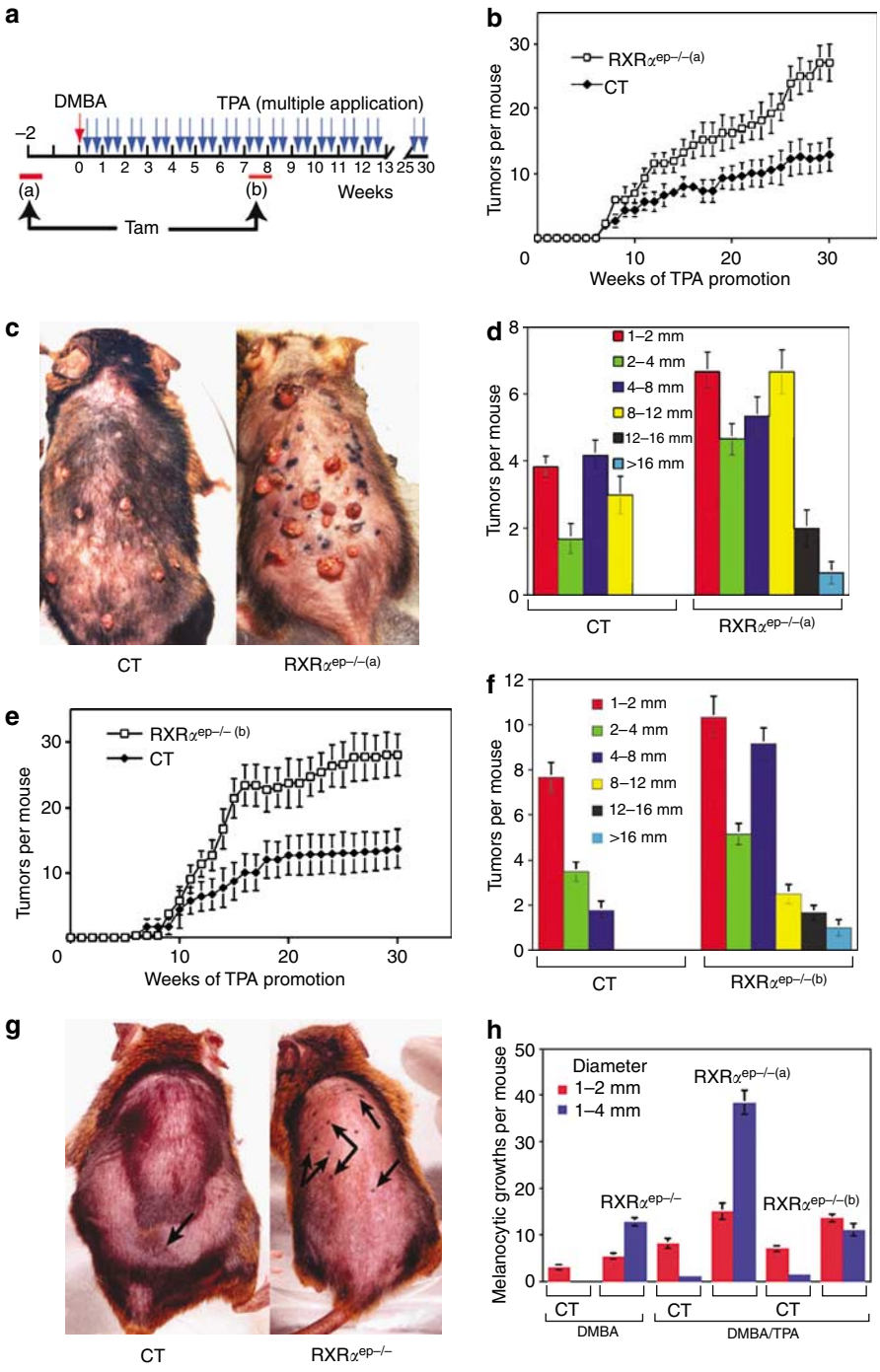

Figure 1. DMBA/TPA-induced epidermal tumors and MGs in RXRα$^{ep-/-}$ mice. (**a**) Timing of Tam-induced RXRα ablation in epidermal keratinocytes, and DMBA/TPA-induced tumorigenesis. Tam-treatment was started either 18 days before (''a'') or 8 weeks after (''b'') topical DMBA application. (**b**) Formation of epidermal tumors in DMBA/TPA-treated CT (K14-Cre-ER$^{T2(0/0)}$/RXRα$^{L2/L2}$) and RXRα$^{ep-/-(a)}$ mice. (**c**) Dorsal view of CT (left) and RXRα$^{ep-/-(a)}$ (right) mice after 25 weeks of DMBA/TPA treatment. (**d**) Epidermal tumor length distribution in CT and RXRα$^{ep-/-(a)}$ mice, after 30 weeks of DMBA/TPA treatment. (**e**) Formation of epidermal tumors in DMBA/TPA-treated CT and RXRα$^{ep-/-(b)}$ mice in which RXRα was ablated in keratinocytes during TPA promotion. (**f**) Epidermal tumor length distribution in CT and RXRα$^{ep-/-(b)}$ mice, after 30 weeks of DMBA/TPA treatment. (**g**) Dorsal view of CT (K14-Cre-ER$^{T2(0/0)}$/RXRα$^{L2/L2}$) (left) and RXRα$^{ep-/-}$ (right) mice 25 weeks after DMBA treatment. Arrows point to MGs. (**h**) Size distribution of MGs (as indicated), 30 weeks after DMBA or DMBA/TPA treatments. Values are expressed as mean ± SEM ($n = 7$).

early stages of malignant conversion (Nischt *et al.*, 1988, and references therein; Rundhaug *et al.*, 1997). Immunohistochemistry of tumors (8–16 mm in length) taken 22 weeks after the start of DMBA/TPA treatment was performed using keratin K5, K1, K10, and K13 antibodies (Figure 2a). K5, ''normally'' selectively expressed in basal cell layer persisted throughout suprabasal layers of highly stratified tumors from CT and RXRα$^{ep-/-(a)}$ mice, but higher K5 levels were found in

RXRα$^{ep-/-(a)}$ tumors (Figure 2a and b). K1 and K10, expressed in suprabasal cell layers of >70% of CT tumors (Figure 2c and e), were expressed in <25% of RXRα$^{ep-/-(a)}$ tumors (Figure 2d and f). Tumors expressing K13 were 3–4 times more frequent in RXRα$^{ep-/-(a)}$ mice, and more than 70% of the tumor area was stained (15% in CT mice; Figure 2g and h, and data not shown).

Upregulation of α6 and β4 integrins (normally expressed in basal cells and mainly found in basement membrane), and their presence into several cell layers above the basement membrane, are associated with a high risk of malignant conversion (Tennenbaum *et al.*, 1993). α6 and β4 Integrin expression was much stronger in RXRα$^{ep-/-(a)}$ tumors and extended into suprabasal layers (Figure 2i and j, and not shown), whereas restricted to the basal layer in >90% of CT tumors. Furthermore, 70% of RXRα$^{ep-/-(a)}$ tumors exhibited increased immunostaining of CD31 which labels endothelial intercellular junctions (Figure 2k and l, and data not shown). Taken together, these data indicate that DMBA/TPA-induced tumors exhibit a higher malignant progression in RXRα$^{ep-/-}$ mice.

DMBA/TPA-induced tumors degenerate at a low rate into SCC, but not into basal cell carcinoma (Yuspa, 1998, and references therein) (see Table 1). Nine tumors from each of 6 CT and 6 RXRα$^{ep-/-(a)}$ mice were examined. After 25 weeks of DMBA/TPA treatment, most of CT tumors were benign papillomas characterised by skin folds integrated by a core of connective tissue and lined by an acanthotic, hyperkeratotic, stratified squamous epithelium (Figure 2m, and data not shown). 20% of the papillomas exhibited basal cell hyperplasia (Figure 2n), 3% displayed *in situ* carcinoma (not shown), and foci of early SCC were observed 30 weeks after tumor initiation in ~14% of the tumors (Figure 2o, Table 1, and not shown). In contrast, 25 weeks after initiation, ~30% of RXRα$^{ep-/-(a)}$ papillomas exhibited basal cell hyperplasia (Figure 2q), 10% displayed *in situ* carcinoma (not shown), and 40% showed foci of SCC (Figure 2r). Five weeks later, 46% of these tumors had progressed to differentiated and undifferentiated SCC with extensive local invasion into the dermis and underlying muscles (Figure 2s–v, Table 1, and not shown). Well-differentiated SCC (Figure 2s), moderately differentiated SCC (Figure 2t), as well as poorly differentiated SCC (Figure 2u), were observed in 30–45% of the tumors (Table 1). Highly aggressive anaplastic SCC (~3%; Figure 2v) and basal cell carcinoma (~6%;Figure 2w and x) were also found in RXRα$^{ep-/-(a)}$, but never in CT mice (Table 1).

Generation of invasive human-melanoma-like MGs in skin of RXRα$^{ep-/-}$ mice upon DMBA/TPA treatment

A single DMBA application to WT skin induces the formation of nevi (benign MGs) that become visible after 10–12 weeks, and are characterized by subepidermal accumulation of melanocytes (Epstein, 1992 and references therein; Husain *et al.*, 1991). Interestingly, a single DMBA treatment of RXRα$^{ep-/-}$ mice resulted in ~7-fold increase in the number of MGs which were generally larger than those of CT (Figure 1g and h, and not shown). Multiple TPA applications after DMBA initiation further increased the number of MGs in CT and mutant mice (Figure 1h; compare also Figure 1c and g, and not shown). After 30 weeks of DMBA/TPA treatment, ~10 nevi were observed per CT mouse (diameter below 2 mm), whereas >50 MGs were found per RXRα$^{ep-/-(a)}$ mouse (75% with a diameter over 2 mm and 7% with a diameter over 4 mm; see Figure 1h, and data not shown). When RXRα was ablated 8 weeks after DMBA treatment (Figure 1a), the number of MGs was ~3-fold higher in RXRα$^{ep-/-(b)}$ than in CT mice, and about 40% of them had a diameter >2 mm (Figure 1h). Thus both MG initiation and promotion might be increased upon ablation of RXRα.

Histological analysis of DMBA or DMBA/TPA-induced MGs showed an accumulation of melanocytes in CT dermis, whereas a larger number of densely melanin-laden melanocytes was seen in both RXRα$^{ep-/-}$ dermis and hypodermis (Figure 3a and b). Most interestingly, 15–20% of the 2–4 mm MGs, and ~95% of >4 mm MGs from RXRα$^{ep-/-}$ mice exhibited a vertical invasion of the underlying musculature (Figure 3b and data not shown). However, in most cases the basement membrane of RXRα$^{ep-/-}$ mice appeared intact and only few melanocytes (1–2%) were found in both CT and RXRα$^{ep-/-}$ epidermis (Figure 3a and b). As previously reported (Ghadially, 1997), electron microscopy revealed that melanocytes from CT nevi contained mainly stage III and IV melanosomes (Figure 3c and e, and not shown). On the other hand, most of the melanocytes present in RXRα$^{ep-/-}$ MGs showed a ~10-fold increase in melanosomes containing melanin granules, and their nuclei were more elongated (Figure 3d and f). Moreover, in ~40% of the RXRα$^{ep-/-}$ melanocytes, 40–50% of the melanosomes had a variable electron opacity with a distinct substructure, a characteristic of human melanoma cells (Ghadially, 1997) (Figure 3d and f, arrows, and not shown). In addition, the membrane of a number of melanosomes was disrupted and smaller melanin granules were scattered in the cellular matrix (Figure 3f, and not shown). In rare cases, oval shaped vesicles contained a longitudinally oriented component made of rods, which is characteristic of human melanoma cells (Figure 3g; Ghadially, 1997). Taken together, these results indicate the formation of human melanoma-like MGs in the skin of RXRα$^{ep-/-}$ mice. However, we failed to detect in MGs of either CT or RXRα$^{ep-/-}$ mice the N-Ras and BRAF mutations which have been found in human melanoma and nevi (see Supplementary Material).

To demonstrate that RXRα was not ablated in MGs, PCR genotyping was performed on DNA isolated from large nevi of CT and RXRα$^{ep-/-}$ mice (Materials and Methods). RXRα L2 alleles, but no RXRα L$^-$ alleles, were found in RXRα$^{ep-/-}$ melanocytic tumors induced by DMBA or DMBA/TPA, whereas only L$^-$ alleles were found in their surrounding epidermis (Figure 3h). Thus the formation of nevi and melanomas in DMBA-treated RXRα$^{ep-/-}$ mice is associated with RXRα ablation in keratinocytes, but not in melanocytes. TPA-induced promotion of DMBA-initiated epidermal tumors can be suppressed by inhibitors of activator protein-1 activity, such as the synthetic glucocorticoid fluocinolone acetonide (FA) (Huang *et al.*, 1997). Topical FA application similarly prevented the appearance of epidermal tumors in 100% of

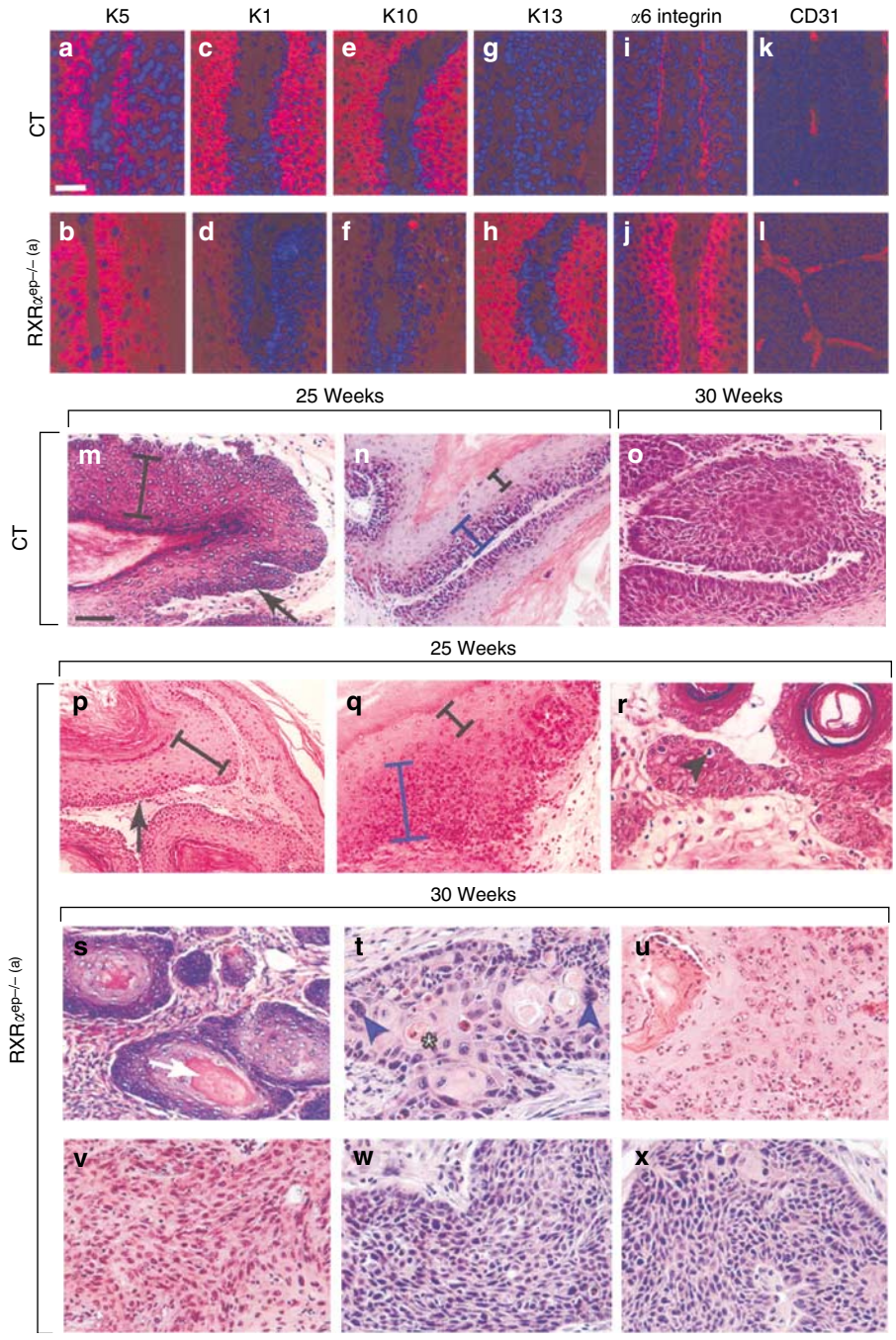

Figure 2. Immunohistochemical and histological analyses of skin tumors induced by DMBA/TPA treatment in RXRα$^{ep-/-}$ mice. (**a**) Representative sections of tumor biopsies from (**a**, **c**, **e**, **g**, **i**, **k**) CT and (**b**, **d**, **f**, **h**, **j**, **l**) RXRα$^{ep-/-(a)}$ mice (after 22 weeks of DMBA/TPA treatment), stained with antibodies against (**a**, **b**) K5, (**c**, **d**) K1, (**e**, **f**) K10, (**g**, **h**) K13, (**i**, **j**) α6 integrin, and (**k**, **l**) CD31. Red corresponds to antibody staining and blue to DNA 4′,6-diamidino-2-phenylindole, dihydrochloride staining. Bar = 25 μm. (**m**–**o**) Representative hematoxylin- and eosin-stained 5-μm-thick paraffin sections from CT biopsies taken after (**m**, **n**) 25 and (**o**) 30 weeks of DMBA/TPA treatment. (**m**) Suprabasal cell hyperplasia (acanthosis) within a benign papilloma; (**n**) basal cell hyperplasia within a benign papilloma; (**o**) focus of early SCC. (**p**–**x**) Representative hematoxylin- and eosin-stained 5-μm-thick paraffin sections from RXRα$^{ep-/-}$ tumor biopsies taken after (**p**–**r**) 25 and (**s**–**x**) 30 weeks of DMBA/TPA treatment. (**p**) suprabasal cell hyperplasia within a benign papilloma; (**q**) basal cell hyperplasia within a benign papilloma; (**r**) focus of early SSC; (**s**) well differentiated (note keratin pearls (white arrow) with keratinization in the center), (**t**) moderately differentiated (cells are markedly irregular in shape and size, and nuclei are hyperchromatic (blue arrowhead) with prominent nucleoli; note internal keratinization (asterisk)), and (**u**) poorly differentiated (note pleiomorphic cancer cells with abnormal nuclei and prominent nucleoli) SCC; (**v**) anaplastic SCC with spindle-shaped cells and absence (or minimal) keratinization; (**w**, **x**) basal cell carcinoma with densely packed basophilic cells with hyperchromatic nuclei resembling those of epidermal basal cells. Hyperplastic basal (blue brackets) and suprabasal (black brackets) cell layers in (**n**, **q**) and (**m**, **n**, **p**, and **q**) are indicated. Black arrows in (**m**, **p**) point to basal cell layers; white arrow in (**s**) points to a keratin pearl; black and blue arrowheads in (**r** and **t**), point to a mitosis and abnormal nuclei, respectively; asterisk in (**t**) indicates internal cell keratinization. Bar in (**m**) 33 μm for (**m**, **n**, **p**, and **q**); 22 μm for (**o**) and (**r**–**x**).

Table 1. Incidence of malignant progression in papillomas generated by a 30-week DMBA/TPA treatment of mice selectively lacking either RXRα or PPARγ in epidermal keratinocytes, or $VDR^{-/-}$ null mice

	Papillomas			Papillomas with SCC				Papillomas with BCC
Mice	Benign	With *in situ* carcinoma	With FC	Well differentiated	Moderately differentiated	Poorly differentiated	Anaplastic carcinomas	
Control	29/35 (83%)	1/35 (3%)	5/35 (14%)	0/35	0/35	0/35	0/35	0/35
$RXR\alpha^{ep-/-}$	11/35 (31%)	1/35 (3%)	4/35 (11%)	11/35 (31%)	3/35 (9%)	2/35 (6%)	1/35 (3%)	2/35 (6%)
$PPAR\gamma^{ep-/-}$	25/41 (68%)	0/41	2/41 (5%)	7/41 (17%)	0/41	2/41 (5%)	1/41 (2.5%)	1/41 (2.5%)
$VDR^{-/-}$	38/40 (95%)	0/40	2/40 (5%)	0/40	0/40	0/40	0/40	0/40

BCC, basal cell carcinoma; DMBA, 7,12-dimethyl-benz[a]anthracene; FC, foci of early squamous cell carcinoma; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid-X-receptor; TPA, 12-*O*-tetradecanoylphorbol-13 acetate.
Epidermal tumors obtained 30 weeks after DMBA/TPA application from six controls, six $RXR\alpha^{ep-/-(a)}$, six $PPAR\gamma^{ep-/-(a)}$ and six $VDR^{-/-}$ mice were analyzed and histologically classified, as indicated.

DMBA/TPA-treated CT (K14-Cre-$ER^{T2(0/0)}$/$RXR\alpha^{L2/L2}$ mice, ($n=10$)) and $RXR\alpha^{ep-/-}$ mice ($n=15$) while, strikingly, it did not affect the increased generation of MGs and their malignant progression in $RXR\alpha^{ep-/-}$ mice when compared to CT mice (Figure 4, compare with Figure 1c; and data not shown).

Selective ablation of PPARγ in keratinocytes enhances DMBA/TPA-induced epidermal tumorigenesis

To investigate whether the RXRα tumor suppressive effect could be due to events controlled by RXRα/PPARγ heterodimers, $PPAR\gamma^{ep-/-}$ (for epidermal keratinocyte-selective PPARγ-null genotype) mice, as well as their CT littermates ($PPAR\gamma^{L2/L2}$, which carry two PPARγ L2 floxed alleles (Imai *et al.*, 2004), were obtained by Tam administration to K14-Cre-ER^{T2}/$PPAR\gamma^{L2/L2}$ and $PPAR\gamma^{L2/L2}$ littermates, respectively. Unlike $RXR\alpha^{ep-/-}$ and $VDR^{-/-}$ mice (see Li *et al.*, 2000 and Figure 1), $PPAR\gamma^{ep-/-}$ mice did not exhibit an alopecia. Furthermore, in contrast to $RXR\alpha^{ep-/-}$ mice, $PPAR\gamma^{ep-/-}$ mice showed only a modest increase in keratinocyte proliferation 6–8 weeks after Tam-induced ablation (≈15% when compared to CT mice, as determined from IHC staining of the Ki67 proliferation marker; not shown), and did not exhibit abnormal interfollicular epidermis differentiation, nor skin inflammatory reaction (Mao-Qiang *et al.*, 2004, and our unpublished data).

Starting 2 weeks after Tam administration (as in Figure 1a), CT and $PPAR\gamma^{ep-/-(a)}$ female mice were subjected to DMBA/TPA treatment. All mice developed epidermal tumors, but their number was 2-fold increased in $PPAR\gamma^{ep-/-(a)}$ mice, and they were generally larger than those of CT (Figure 5a–c). To investigate whether the effect of PPARγ on tumorigenesis was exerted on initiation and/or promotion steps, PPARγ was selectively ablated by Tam administration 7 weeks after the start of the DMBA/TPA treatment ($PPAR\gamma^{ep-/-(b)}$ mice; as in Figure 1a). Epidermal tumors appeared ∼1 week later in both CT and $PPAR\gamma^{ep-/-(b)}$ mice. After 30 weeks, their number was again approximately 2-fold increased in $PPAR\gamma^{ep-/-(b)}$ mice (Figure 5d and e), indicating that PPARγ ablation in epidermal keratinocytes stimulates the promotion, but not the initiation step of tumorigenesis. Treatment with TPA alone was not tumorigenic (not shown). Histological analysis of 40 tumors from $PPAR\gamma^{ep-/-(a)}$ mice, 30 weeks after tumor promotion, revealed that 68% of these tumors were benign papillomas, whereas 24.5% had progressed to SCC, and 2.5% to basal cell carcinoma (Table 1).

MGs also appeared on DMBA/TPA-treated $PPAR\gamma^{ep-/-(a)}$ and $PPAR\gamma^{ep-/-(b)}$ mice (not shown). However, their number was similar in $PPAR\gamma^{ep-/-}$ and CT mice (Figure 5f), and their analysis (MGs ∼2 mm in diameter) showed dermal melanin-laden melanocytes similar to those observed in benign nevi (see above), with no evidence of malignant progression (not shown).

DMBA/TPA treatment of VDR-null mutant mice does not enhance epidermal tumorigenesis, but increases the formation of MGs

Like $RXR\alpha^{ep-/-}$ mice, VDR-null ($VDR^{-/-}$) mice (Yoshizawa *et al.*, 1997) develop an alopecia, which suggested that RXRα/VDR heterodimers are indispensible during hair cycling (Li *et al.*, 2001, and references therein; Li *et al.*, 2000). It has also been reported that vitamin D3, the active VDR ligand, can induce both cell cycle arrest and differentiation in vitro of human cell lines derived from SCC (Niles, 1995) and melanoma (Yudoh *et al.*, 1999), and reduce tumor formation in the two-step chemical tumorigenesis mouse model (Chida *et al.*, 1985; Kensler *et al.*, 2000). Eight-to-nine-week-old VDR-null mutant and WT CT females were DMBA/TPA-treated to investigate whether VDR could be involved in the tumor-suppressive effect of RXRα. Seven to eight weeks after the start of TPA treatment, papillomas developed similarly in all CT and mutant mice (Figure 6a and b, and data not shown), although they were in general smaller in $VDR^{-/-}$ than in CT mice (Figure 6a–c). Histological analysis of 40 tumors, 30 weeks after DMBA initiation revealed that, unlike $RXR\alpha^{ep-/-}$ mutants, 95% of the papillomas were benign, whereas the others only exhibited foci of early SCC (Table 1, and data not shown).

MGs also appeared on DMBA and DMBA/TPA-treated skin of $VDR^{-/-}$ mice, and their number was strikingly high to

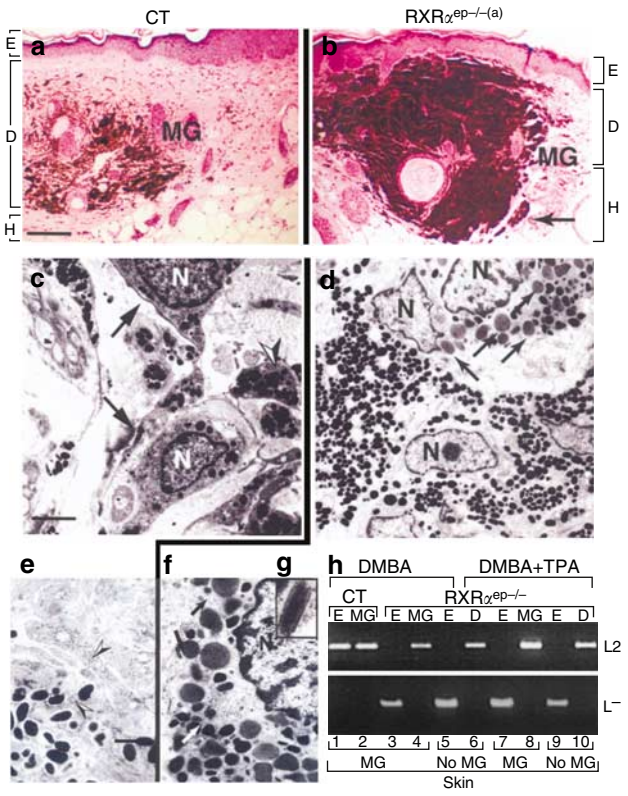

Figure 3. DMBA/TPA induced human melanoma like MGs in RXRα$^{ep-/-}$ mice. (**a**–**g**) Histological and ultrastructural analyses of MGs from CT and RXRα$^{ep-/-(a)}$ mice. (**a** and **b**) Hematoxylin and eosin-stained paraffin sections (5 μm thick) of (**a**) CT (K14-Cre-ER$^{T2(0/0)}$/RXRα$^{L2/L2}$) and (**b**) RXRα$^{ep-/-(a)}$ MG containing skin biopsies taken 25 weeks after DMBA/TPA treatment. The arrow in (**b**) points to melanocytic invasion of the hypodermis. E, epidermis; D, dermis; H, hypodermis. Bar = 33 μm. (**c**–**g**) Electron microscopic analyses of (**c** and **e**) CT and (**d**, **f**, and **g**) RXRα$^{ep-/-(a)}$ MGs isolated 25 weeks after DMBA/TPA treatment. Note in the inset (**g**), the oval-shaped subcellular structure containing a longitudinally oriented component made of rods. Arrows in (**c**, **d**, and **f**) point to (**c**) CT ''normal'' dermal melanocytes, and (**d** and **f**) abnormally shaped melanosomes typical of human-melanoma-like MG, respectively. Arrowheads in (**c** and **e**) point towards a melanin-laden melanophage and melanosomes of stage III and IV, respectively. N, nucleus. Bars = 2.5 μm for (**c** and **d**); 1 μm for (**e** and **f**) and 0.25 μm for the (**g**) inset. (**h**) RXRα is not ablated in MGs from RXRα$^{ep-/-}$ mice. The presence of RXRα L2 and L$^-$ alleles was analysed by PCR in DNA from epidermis (E), dermis (D), isolated from MG-free skin biopsies (NoMG) and MG from CT (lanes 1 and 2, K14-Cre-ER$^{T2(0/0)}$/RXRα$^{L2/L2}$) and RXRα$^{ep-/-}$ (lanes 3–10) mice, 25 weeks after DMBA or DMBA/TPA treatment, as indicated. The position of the RXRα L2 and L$^-$ alleles is indicated.

reach ~70 MGs after 30 weeks of TPA application (instead of ~10 MGs in CT mice, Figure 6d). In both cases, histological analysis of 30 MGs from CT and VDR$^{-/-}$ mice showed the presence of dermal melanin-laden melanocytes (Figure 6e and f, and data not shown), and electron microscopy revealed that they contained mostly stage III and stage IV melanosomes which are characteristics of benign nevi (Figure 6g and h). However, the number of melanosomes was approximately 2-fold increased in ~10% of the melanocytes present in VDR$^{-/-}$ MGs (Figure 6g and h, and data not shown).

DISCUSSION

Keratinocytic RXRα and PPARγ act as tumor modifiers of both promotion and malignant progression of epidermal tumors induced by topical DMBA/TPA treatment

Our results show that RXRα acts as a tumor modifier of both the TPA-induced promotion of benign papilloma-like tumors and their subsequent progression to malignancy, whereas the DMBA-induced initiation step which, results in H-ras activation is not affected. TPA-induced tumor necrosis factor-α expression in keratinocytes, which results in their hyperproliferation and a skin inflammation, is known to be critical for tumor promotion (Moore *et al.*, 1999; Suganuma *et al.*, 1999), and to be suppressed by inhibitors of activator protein-1 activity, such as the glucocorticoid FA (Huang *et al.*, 1997). As keratinocyte hyperproliferation and skin inflammation are also generated upon RXRα ablation in epidermal keratinocytes (Li *et al.*, 2000, 2001), and given the association between chronic inflammation and cancer (Coussens and Werb, 2002), the question arises whether the promoting effect of RXRα ablation could correspond to an increase in tumor necrosis factor-α production. No such increase was found, ruling out that it could be responsible for the enhancement of TPA-induced tumor promotion observed upon RXRα ablation, even though this enhancement was also suppressed by administration of FA (data not shown).

In quest of partners with which RXRα may heterodimerize in keratinocytes to modify the TPA-induced promotion and malignant progression of DMBA-initiated epidermal tumors, mice ablated for NRs known to be expressed in the skin were also subjected to the same DMBA/TPA treatment. PPARα (Sheu *et al.*, 2002), PPARβ(δ) (Schmuth *et al.*, 2004) and PPARγ (Mao-Qiang *et al.*, 2004) were selected as their

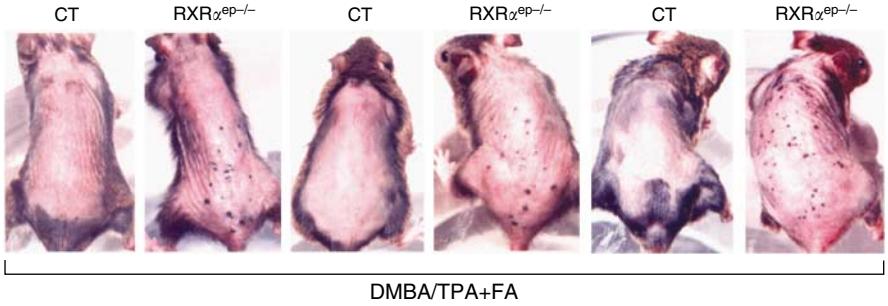

Figure 4. Effect of FA treatment on DMBA/TPA-induced epidermal tumors and MGs. Dorsal view of three CT (K14-Cre-ER$^{T2(0/0)}$/RXRα$^{L2/L2}$ mice) and three RXRα$^{ep-/-}$ mice after 25 weeks of DMBA/TPA and FA co-treatment of the shaved area. These mice are representative of 10 CT and 15 RXRα$^{ep-/-}$ mice.

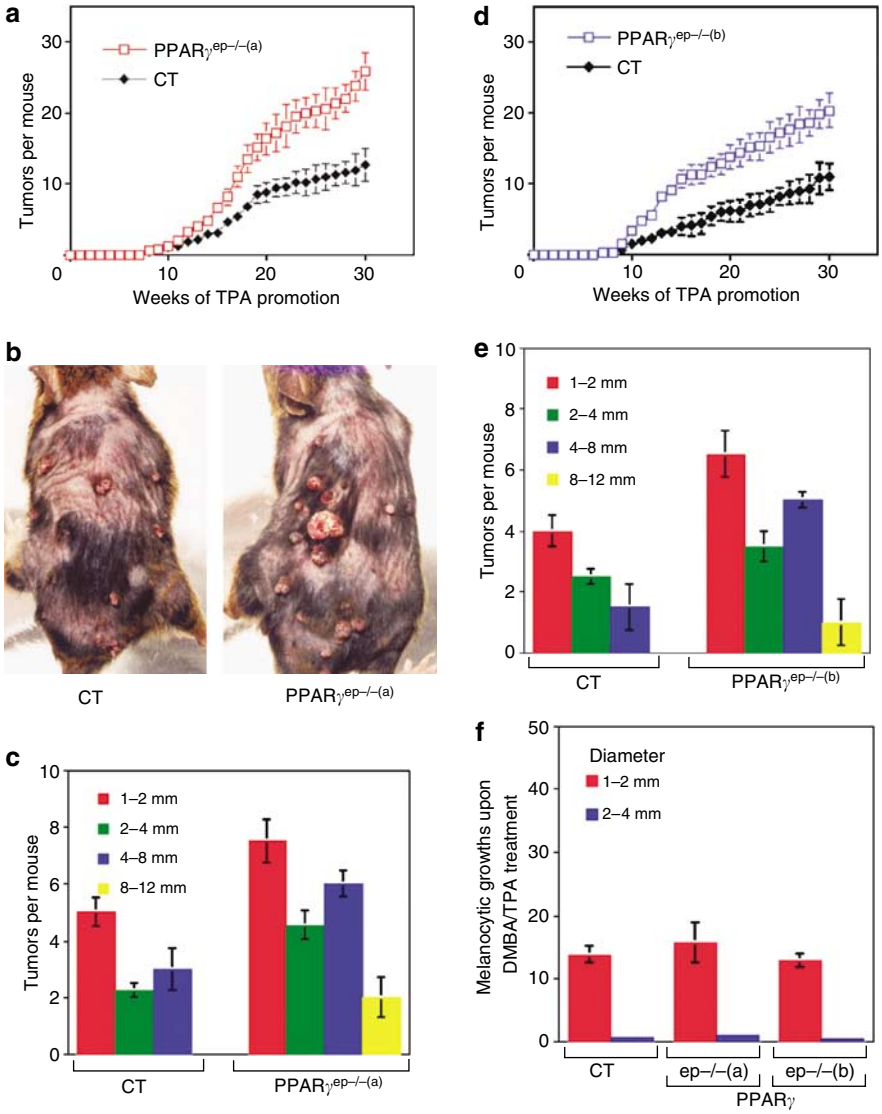

Figure 5. DMBA/TPA-induced epidermal tumors and MGs in PPARγ$^{ep-/-(a)}$ and PPARγ$^{ep-/-(b)}$ mice. (**a**) Formation of epidermal tumors in DMBA/TPA-treated CT and PPARγ$^{ep-/-(a)}$ mice. (**b**) Dorsal view of CT (K14-Cre-ER$^{T2(0/0)}$/PPARγ$^{L2/L2}$) (left) and PPARγ$^{ep-/-(a)}$ (right) mice 25 weeks after the start of DMBA/TPA treatment. (**c**) Length distribution of epidermal tumors in CT and PPARγ$^{ep-/-(a)}$ mice as measured after 25 weeks of DMBA/TPA treatment. (**d**) Formation of epidermal tumors in DMBA/TPA-treated PPARγ$^{ep-/-(b)}$ mice in which PPARγ was ablated in keratinocytes during TPA promotion. (**e**) Length distribution of epidermal tumors in CT and PPARγ$^{ep-/-(b)}$ mice, after 25 weeks of TPA treatment. (**f**) Size distribution (diameter) of MGs in CT, PPARγ$^{ep-/-(a)}$, and PPARγ$^{ep-/-(b)}$ mice. Values are expressed as mean ± SEM ($n = 7$ mice).

activator ligands are known to inhibit TPA-induced cutaneous inflammation. The selective ablation of PPARβ in basal keratinocytes of adult mouse skin had no significant effect on DMBA/TPA-induced tumorigenesis (our unpublished data). As it has been reported that this tumorigenesis is enhanced in PPARβ-null mice (Kim *et al.*, 2004), PPARβ might exert its tumor modifier activity in non-keratinocyte cells, and /or its expresssion in keratinocytes of the developing epidermis might prevent tumorigenesis. In contrast, in PPARγ$^{ep-/-}$ mice ablated for PPARγ in keratinocytes, the increased formation of epidermal tumors and their elevated progression to carcinomas, were similar to those observed in RXRα$^{ep-/-}$ mice, even though, in contrast to RXRα, the selective ablation of PPARγ in keratinocytes does not result in skin inflammation (Mao-Qiang *et al.*, 2004, and our unpublished results). Thus, PPARγ is likely to be the RXRα heterodimerization partner in the cell-autonomous process(es) that CT(s) tumor promotion and malignant progression in DMBA/TPA-treated mice. Interestingly, germ line null mutations of PPARα or LXRβ, whose activations inhibit TPA-induced cutaneous inflammation (Fowler *et al.*, 2003), similarly resulted in 1.5 to 2-fold increased formation of DMBA/TPA-induced tumors (our unpublished results). Keratinocyte-selective ablation PPARα and LXRβ is required to determine whether RXRα/PPARα and RXRα/LXRβ heterodimers also act as cell-autonomous epidermal tumor modifiers. On the other hand, germline null mutations of VDR (this study), as well as single or compound RARα and RARγ or RARβ keratinocyte-selective

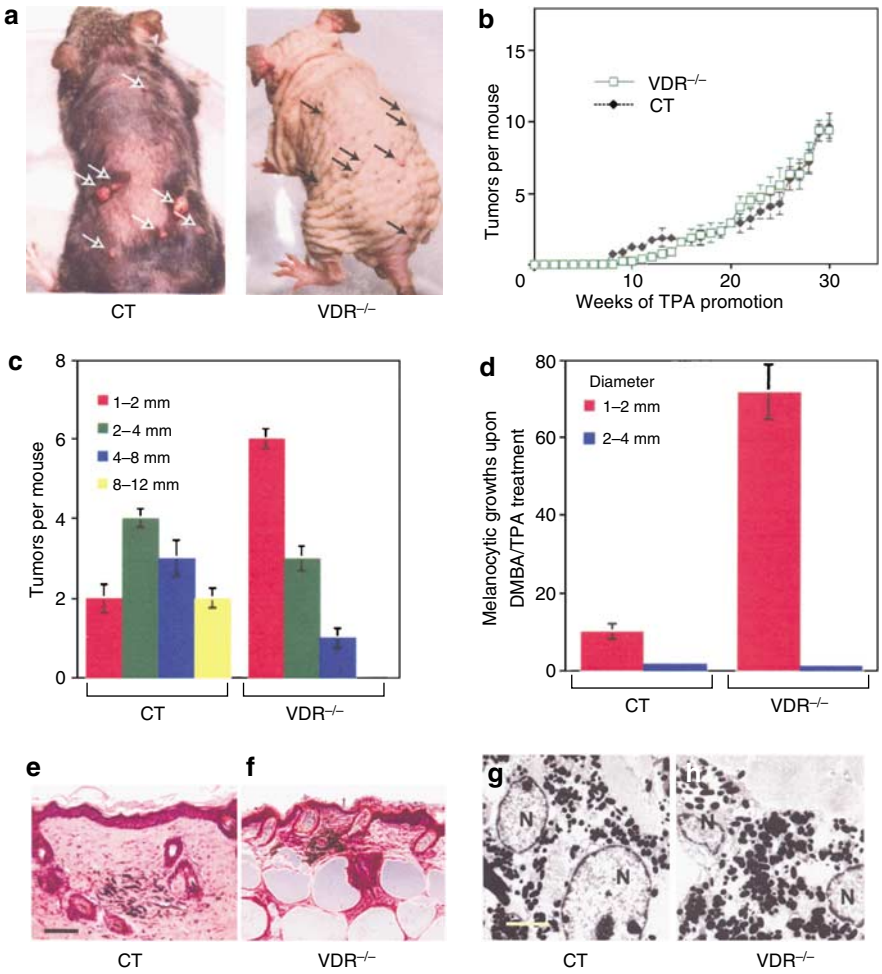

Figure 6. DMBA/TPA-induced epidermal tumors and MGs in VDR-null mice. (**a**) Dorsal view of CT ($VDR^{+/+}$) (left) and VDR-null ($VDR^{-/-}$, right) mice 25 weeks after the start of DMBA/TPA treatment. Arrows point to papilloma-like epidermal tumors. (**b**) Formation of epidermal tumors in DMBA/TPA-treated CT and $VDR^{-/-}$ mice. Values are expressed as mean ± SEM ($n = 7$ mice). (**c**) Length distribution of epidermal tumors in CT and $VDR^{-/-}$ mice, 30 weeks after the start of the DMBA/TPA treatment. Values are expressed as mean ± SEM ($n = 7$ mice). (**d**) Size distribution of MGs in CT and $VDR^{-/-}$ mice, 30 weeks after the start of TPA treatment. Values are expressed as mean ± SEM ($n = 7$ mice). (**e**, **f**) Hematoxylin- and eosin-stained paraffin sections (5 μm thick) of (**e**) CT and (**f**) $VDR^{-/-}$ MGs in skin biopsies taken after 25 weeks of DMBA/TPA treatment. Bar = 33 μm. (**g**, **h**) Electron microscopic analysis of (**g**) CT and (**h**) $VDR^{-/-}$ MGs taken after 25 weeks of DMBA/TPA treatment. N, nucleus. Bar = 2.5 μm.

mutations, had no significant effect on DMBA/TPA-induced epidermal tumorigenesis (our unpublished data).

The formation of DMBA-induced dermal benign MGs and their malignant progression to invasive human-melanoma-like MGs upon keratinocyte-selective ablation of RXRα involves a paracrine signalling(s).

In contrast to human skin melanocytes, which are mainly located in the interadnexal epidermis, mouse skin melanocytes drastically decrease in interfollicular epidermis from the fourth day after birth. In adult mice, few melanocytes are present in the dermis, whereas the bulk of them are confined to hair follicles (Hirobe, 1984; Hardy, 1992; Yoshida *et al.*, 1996). A single topical application of DMBA to mouse skin induces the formation of benign MGs that become visible as nevi (dermal accumulation of melanocytes) (Husain *et al.*, 1991). Our data show that nevi are increased in number and size upon further RXRα ablation in epidermal keratinocytes of adult mice. Importantly, and as expected, RXRα ablation could not be detected in isolated MGs, whereas it was readily revealed in MG-free epidermis. In both CT and $RXR\alpha^{ep-/-}$ mice, multiple TPA applications further increase the number of nevi. Furthermore, irrespective of TPA treatment, a significant proportion of MGs from $RXR\alpha^{ep-/-}$ mice exhibited a malignant progression to invasive human-melanoma-like tumors. In agreement with the report of Husain *et al.* (1991), this malignant progression was never found in CT mice. Therefore, the genetically DMBA-initiated proliferation of melanocytes and their malignant progression must be controlled by paracrine/juxtacrine signals, which emanate from keratinocytes, and are regulated by RXRα.

The identity of the NR partner(s) which heterodimerize(s) with RXRα in keratinocytes to directly or indirectly down-

regulate the generation of nevi and their malignant progression to human-melanoma-like tumors is presently unknown. No increase in DMBA-induced nevi formation and malignant progression was found in skin of mice selectively ablated for possible RXRα partners in keratinocytes (PPARβ, PPARγ, RARα, RARγ, RARβ, RARα and γ) or in the germ line (PPARα, and LXRβ) (this study and our unpublished results). Furthermore, VDR cannot be the partner of RXRα in heterodimers, which in keratinocytes are instrumental in preventing the overproduction of nevi (melanocytosis), even though we found a marked increase in benign MGs in VDR-null mutants, which however did not progress to malignancy. Indeed, keratinocyte-selective-ablation of VDR did not result in melanocytosis (our unpublished results). Thus, our results strongly suggest that RXRα heterodimerized in keratinocytes with as yet unidentified NR partner(s) prevents the proliferation, migration and malignant progression of DMBA-initiated melanocytes through downregulation of paracrine pathways.

Taken together our present study demonstrates that keratinocytic RXRα plays a key role in modulating the formation and malignant transformation of DMBA/TPA-induced epidermal skin tumors, as well as in the generation of DMBA-induced MGs and their malignant progression to human-melanoma-like MGs. Several lines of evidence support the conclusion that distinct RXRα/NR-mediated mechanisms are involved upon DMBA/TPA treatment to block (i) the generation of paracrine signal(s) that trigger(s) the proliferation of melanocytes and their possible malignant transformation, and cannot be prevented by FA co-treatment, and (ii) the occurrence of cell-autonomous events that lead to formation and malignant progression of epidermal tumors, and can be prevented by FA co-treatment. First of all, the tumor promoter TPA is indispensable to generate epidermal, but not melanocytic tumors in adult RXRα$^{ep-/-}$ mice in which RXRα is selectively ablated in epidermal keratinocytes. Secondly, in DMBA/TPA-treated RXRα$^{ep-/-}$mice, the topical application of the synthetic glucocorticoid FA strikingly prevents the occurrence of epidermal tumors, but not the generation of melanocytic tumors (MGs and human-like-melanomas). Thirdly, in keratinocytes, RXRα/PPARγ heterodimers appear to mediate cell-autonomous suppressive effects on epidermal tumorigenesis and malignant progression, whereas they do not play any role in preventing the formation and malignant progression of MGs. Thus, through its multiple NR partnerships, RXRα in keratinocytes plays a paramount role in the control of chemically induced tumorigenesis, not only in the epidermis, but also in melanocytes. This emphasizes the crucial role of keratinocytes in skin tumorigenesis, and raises the possibility that they could play a similar role in the generation of UV-induced tumors, notably in the formation and malignant progression of melanocytic tumors.

MATERIALS AND METHODS

These procedures are provided in Supplementary Material.

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Data. Materials and Methods, additional results and references.

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