Physiological and retinoid-induced proliferations of epidermis basal keratinocytes are differently controlled

Benoit Chapellier, Manuel Mark, Nadia Messaddeq, Cécile Calléja, Xavier Warot, Jacques Brocard, Christelle Gérard, Mei Li, Daniel Metzger, Norbert B.Ghyselinck and Pierre Chambon¹

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 10142, 67404 Illkirch Cedex, CU de Strasbourg, France

¹Corresponding author
e-mail: chambon@igbmc.u-strasbg.fr

N.B.Ghyselinck and P.Chambon contributed equally to this work

To investigate the roles of retinoic acid (RA) receptors (RARs) in the physiology of epidermis that does not express RAR β , conditional spatio-temporally controlled somatic mutagenesis was used to selectively ablate RAR α in keratinocytes of RAR γ -null mice. Keratinocyte proliferation was maintained in adult mouse epidermis lacking both RAR α and RAR γ , as well as in RAR β -null mice. All RAR-mediated signalling pathways are therefore dispensable in epidermis for homeostatic keratinocyte renewal. However, topical treatment of mouse skin with selective retinoids indicated that RXR/RAR γ heterodimers, in which RXR transcriptional activity was subordinated to that of its RAR γ partner, were required for retinoid-induced epidermal hyperplasia, whereas RXR homodimers and RXR/RAR α heterodimers were not involved. RA-induced keratinocyte proliferation was studied in mutant mice in which RXR α , RXR β and RAR α , RAR γ , or RXR α and RAR γ genes were specifically disrupted in either basal or suprabasal keratinocytes. We demonstrate that the topical retinoid signal is transduced by RXR α /RAR γ heterodimers in suprabasal keratinocytes, which, in turn, stimulate proliferation of basal keratinocytes via a paracrine signal that may be heparin-binding EGF-like growth factor.

Keywords: conditional somatic mutagenesis/heparin-binding EGF-like growth factor/nuclear receptor/paracrine control/synthetic retinoids

Introduction

The initial observations of abnormal keratinization of various epithelia in vitamin A-deficient rats (Wolbach and Howe, 1925) and humans (Frazier and Hu, 1931) were followed by numerous pharmacological studies with vitamin A derivatives (retinoids), which led to the development of retinoid therapy for several skin diseases (Livrea, 2000). When applied topically on adult skin, retinoic acid (RA, the biologically active metabolite of vitamin A) generates an epidermal hyperplasia that results

from an hyperproliferation of basal keratinocytes leading, upon their vectorial migration towards the skin surface, to thickening of the differentiated suprabasal (spinous and granular) layers (Fisher and Voorhees, 1996). RA treatment also decreases the cohesiveness of the stratum corneum, impairing the adult skin barrier and increasing trans-epidermal water loss (Elias *et al.*, 1981). Similar effects were induced by synthetic retinoids (Chen *et al.*, 1995; Thacher *et al.*, 1997). Although retinoid pharmacological effects have been more studied in skin than in any other tissue, the skin cell-type(s) in which RA regulates gene expression and the network of RA-responsive target genes remain largely unknown.

Retinoids exert their highly pleiotropic effects through two groups of nuclear receptors (NRs), the retinoic acid receptors (RAR α , β and γ) and the retinoid X receptors (RXR α , β and γ), which belong to the NR superfamily of ligand-dependent transcriptional regulators. RARs bind *all trans*- and *9cis*-RA stereo-isomers, whereas RXRs interact exclusively with *9cis*-RA. RARs and RXRs act through binding to *cis*-acting response elements located in the regulatory regions of retinoid-responsive genes, and RARs, like NRs for thyroid hormone (TRs), vitamin D3 (VDR), peroxisome proliferators (PPARs) and several orphan receptors, require heterodimerization with RXRs to function *in vitro* and *in vivo* (Kastner *et al.*, 1995; Chambon, 1996; Morriss-Kay and Ward, 1999).

RXR α , RXR β , RAR α and RAR γ are expressed in epidermis, and RXR α and RAR γ are the predominant receptors (Fisher and Voorhees, 1996). However, their precise function in mediating retinoid effects on epidermis are unknown. Mouse transgenic expression of a dominant-negative (dn) RAR α that subverts wild-type RAR functions has suggested that RARs could be involved in keratinocyte differentiation and RA-induced hyperproliferation (Imakado *et al.*, 1995; Saitou *et al.*, 1995; Xiao *et al.*, 1999). Furthermore, expression of a dnRXR α in epidermal suprabasal layers indicated that RXR α could also be involved in retinoid-induced cell proliferation in adult mouse skin (Feng *et al.*, 1997). However, as dn receptors could artefactually repress the expression of genes that are not 'normal' targets of co-repressor-associated unliganded RXR/RAR heterodimers (Chen and Evans, 1995), and/or interfere through sequestration with a variety of signalling pathways mediated by NRs that heterodimerize with RXRs (Mangelsdorf *et al.*, 1995), the abnormalities exhibited by these transgenic models may not reflect the possible physiological roles of RA in epidermis homeostasis.

Targeted gene disruption through homologous recombination has been used extensively to investigate the physiological functions of retinoid receptors in the mouse. These genetic studies have indicated that RAR α is apparently dispensable for epidermal homeostasis,

whereas RAR γ is involved in minor aspects of granular keratinocyte differentiation (our unpublished data). However, due to functional redundancy amongst retinoid receptors, some functions of RAR α and/or RAR γ in epidermis could have been overlooked (Kastner *et al.*, 1995, 1997; Mascres *et al.*, 1998 and references therein). Unfortunately, compound germ-line disruptions of RAR α and RAR γ lead to lethality before embryonic day (E)11.5 (Wendling *et al.*, 2001), thus precluding any epidermis analysis. Similarly, the possible effect of RXR α knockout on mouse epidermis could not be assessed, as this mutation is lethal at E14.5, i.e. at the onset of epidermal morphogenesis. We have recently circumvented these limitations by using the Cre/loxP technology and a tamoxifen-inducible Cre-ER^T recombinase (Metzger and Chambon, 2001) in order to selectively abrogate RXR α expression in adult mouse keratinocytes. This approach allowed us to demonstrate that RXR α is dispensable for epidermal morphogenesis, but is involved in the control of hair cycling, most probably through RXR α /VDR heterodimers (Li *et al.*, 2000, 2001). We also found that RXR α , heterodimerized with other NRs, could be implicated in the control of interfollicular keratinocyte proliferation and differentiation (Li *et al.*, 2000, 2001). To further investigate the skin function of RAR α , RAR γ and RXR α , we produced mice selectively lacking them in epidermal keratinocytes.

Results

RAR-mediated signalling is dispensable for homeostatic epidermal proliferation

As RAR β was not detected in epidermis (Fisher *et al.*, 1994; see below) and epidermis of RAR β -null mice is apparently normal (Ghyselinck *et al.*, 1997), the cell-autonomous requirement of the RAR-mediated signalling pathway in epidermis homeostasis can be studied in mutant mice selectively lacking RAR α and RAR γ in keratinocytes. To generate such mutants, RAR $\alpha^{L2/L2}$ mice (Figure 1A; Chapellier *et al.*, 2002a) were first crossed with RAR $\gamma^{+/-}$ heterozygotes (Lohnes *et al.*, 1993), and subsequently with K5-Cre-ER^{T(tg/0)} transgenic mice that express the tamoxifen-inducible Cre recombinase specifically in basal keratinocytes (Indra *et al.*, 1999). These crosses generated RAR $\alpha^{L2/L2}$, RAR $\alpha^{L2/L2}$ /RAR $\gamma^{-/-}$ and K5-Cre-ER^{T(tg/0)}/RAR $\alpha^{L2/L2}$ /RAR $\gamma^{-/-}$ animals. These mice were subjected to the tamoxifen (TAM) treatment known to permanently ablate RXR α in epidermis and hair follicle (i.e. RXR $\alpha^{ep-/-}$ mice; Li *et al.*, 2000). Excision at the RAR $\alpha^{L2/L2}$ locus in TAM-treated mice harbouring the K5-Cre-ER^T transgene was efficient, permanent and selective, as RAR α L2 alleles were converted into L⁻ alleles in tail epidermis, no RAR α L2 allele could be recovered in tail epidermis, even 12 months after TAM treatment, and no excision could be detected in dermis (thus yielding RAR $\alpha^{ep-/-}$ mice; Figure 1B; data not shown). To check whether RAR β could be aberrantly expressed in epidermis upon RAR α and/or RAR γ ablation, RAR β RNA levels were determined in skin samples. RAR β 1/3 isoforms were not detected, whereas similar levels of RAR β 2 isoform were present in control and RAR $\alpha^{ep-/-}$ /RAR $\gamma^{-/-}$ mice (Figure 1C). As RAR β is selectively expressed in dermis (Redfern and Todd,

1992; our unpublished data), these data show that it is neither upregulated in dermis nor ectopically expressed in epidermis of RAR $\alpha^{ep-/-}$ /RAR $\gamma^{-/-}$ mice, which therefore exhibit a 'panRAR-null' epidermis (panRAR $\alpha^{ep-/-}$ mice).

Topical RA alters epidermal cell differentiation (Fisher and Voorhees, 1996). However, histology of skin biopsies did not reveal any alteration in panRAR $\alpha^{ep-/-}$ mice (compare Figure 1D with F; and see below), and there was no alteration of keratin 5, 6, 10 and 13 expression (immunohistochemical data not shown). Moreover, electron microscopy of epidermis from panRAR $\alpha^{ep-/-}$ mice did not reveal abnormalities other than those exhibited by RAR $\gamma^{-/-}$ mice (data not shown), indicating that RAR α does not compensate for the loss of RAR γ during keratinocyte differentiation in adult mice.

About 1% of basal keratinocytes were BrdU positive in epidermis of 12-month-old RAR $\alpha^{L2/L2}$ mice (Figure 1G). This low labelling index relative to that observed in younger adults ($5 \pm 2\%$, see below and Table I) is a normal feature of epidermal ageing (Engelke *et al.*, 1997). Similarly, in 12-month-old RAR $\gamma^{-/-}$ and panRAR $\alpha^{ep-/-}$ animals, 1% of basal keratinocytes were BrdU positive (Figure 1H and I). Thus, homeostatic keratinocyte proliferation is maintained for >1 year in absence of RAR α , β and γ in epidermis.

Pharmacological evidence that RXR/RAR γ heterodimers are involved in RA-induced epidermal hyperplasia

Dorsal epidermis of 2- to 3-month-old mice consists of (i) an irregular layer of basal keratinocytes, (ii) suprabasal layers formed by scattered spinous and granular keratinocytes (arrowheads) and (iii) several rows of cornified keratinocytes (Figure 2A). Topical RA treatment causes epidermal thickening due to basal cell hyperproliferation (Fisher and Voorhees, 1996). Accordingly, suprabasal layers were thickened in RA-treated wild-type mice, due to marked increases in numbers of spinous and granular keratinocytes; mitotic figures were frequent in basal keratinocytes (Figure 2B), and the number of basal keratinocytes expressing the proliferation marker Ki67 or incorporating BrdU was ~10-fold higher in RA- than in vehicle-treated epidermis ($56 \pm 5\%$ versus $6 \pm 2\%$; Figure 2C and D; Table I; data not shown). Simultaneous administration of the panRAR antagonist BMS493 prevented RA-induced epidermal hyperplasia, demonstrating that keratinocyte proliferation resulted from RAR activation (Figure 2E; Table I). Interestingly, a panRAR antagonist application on its own had no apparent effect on keratinocyte proliferation (data not shown).

To determine the potency of RAR isotypes in mediating retinoid-induced epidermal hyperplasia, wild-type mice were treated topically with selective ligands for RARs or RXRs. On their own or in association (Figure 2H), the panRXR agonist BMS649 (Figure 2F) and the RAR α -selective agonist BMS753 (Figure 2G) did not alter the percentage of proliferating, Ki67-positive, basal keratinocytes (Table I). In contrast, the number of proliferating keratinocytes was strongly increased by the RAR γ -selective agonist BMS961 (Figure 2I). However, the potency of this latter retinoid was below that of RA, while co-administration of BMS961 and BMS649 fully reproduced the effects of RA (compare Figure 2D, I and J; Table I).

Fig. 1. Conditional mutagenesis of *RARα* in epidermis and homeostatic keratinocyte proliferation. **(A)** Schematic drawing of wild-type *RARα* locus (+), floxed L2 and excised (L⁻) alleles. Black boxes stand for exons 7–9 (E7–E9). Restriction sites and location of the 3' probe are indicated. Sizes of restriction fragments are in kilobases (kb). H, *HpaI*; S, *SacI*. Arrowhead flags represent loxP sites. **(B)** Efficiency of Cre-ER^T-mediated *RARα* gene disruption. Fourteen-week-old mice (genotypes as indicated) received TAM (5 days, 1 mg/day), and were treated again 2, 4 and 6 weeks later. *RARα* L2 and L⁻ alleles were identified on tail epidermis genomic DNA, before and 6 months after TAM injection. M: DNA ladder. **(C)** *RARβ* expression in skin samples. RNase protection assay was performed on total RNA (20 μg) from control (*RARα*^{L2/L2}) and mutant mice (*RARγ*^{-/-} and *RARα*^{ep-/-}/*RARγ*^{-/-}), 6 and 12 months after TAM administration. A tRNA sample and total RNAs from RA-treated F9 teratocarcinoma cells were used as controls. Histone H4 protection was included for quantitation of the RNA samples. **(D–F)** Representative skin semi-thin sections, 12 months after TAM administration (genotypes as indicated). **(G–I)** Representative skin sections labelled with BrdU (white colour), showing proliferation of basal keratinocytes 12 months after TAM administration (genotypes as indicated). Sections were counterstained with DAPI (blue colour). Arrows point to the dermal-epidermal junction. hf, hair follicles. Scale bar (in F and I): 50 μm.

Altogether, these observations indicate that RA-induced skin hyperplasia most probably involves RXR/*RARγ* heterodimers, in which RXR transcriptional activity is subordinated to that of *RARγ*, i.e. liganded RXR is inactive unless its *RAR* partner is itself liganded

(Chambon, 1996). However, these pharmacological experiments do not reveal which cells are retinoid primary targets in epidermis and/or even in dermis, which is known to secrete various growth factors involved in epidermal cell proliferation (Werner and Smola, 2001). We therefore

Table I. Overview of retinoid effects on proliferation and gene expression in the epidermis

Genotype	Treatment	Epidermal status	% BrdU-positive nuclei	% Ki67-positive nuclei	CRABP II mRNA abundance	HB-EGF mRNA abundance
Wild type	none	Resting (control)	5 ± 2	6 ± 2	+	+
Wild type or wild-type-like ^a	RA	RA-stimulated	54 ± 6	56 ± 5	++++	++++
Wild type	RA + BMS493	Antagonism of RA stimulation	ND	6 ± 3	+	+
Wild type	BMS649 (SR11237)	Activation of RXR signalling	ND	10 ± 4	+	+
Wild type	BMS753	Activation of RAR α signalling	ND	7 ± 2	ND	ND
Wild type	BMS753 + BMS649	Activation of RAR α and RXR signalling	ND	9 ± 3	ND	ND
Wild type	BMS961	Activation of RAR γ signalling	ND	35 ± 6	+++	+++
Wild type	BMS961 + BMS649	Activation of RAR γ and RXR signalling	ND	51 ± 8	++++	++++
RAR α ^{-/-}	RA	RAR α -null	50 ± 4	ND	++++	++++
K5-Cre-ER ^{T(tg/0)} /RXR α ^{L2/L2}	RA	RXR α -null in all layers (RXR α ^{ep-/-})	12 ± 4	ND	++	++
K5-Cre-ER ^{T(tg/0)} /RXR α ^{L2/L2} /RAR α ^{-/-}	RA	RXR α -null in all layers and RAR α -null (RXR α ^{ep-/-} /RAR α ^{-/-})	13 ± 4	ND	ND	ND
RAR γ ^{-/-}	RA	RAR γ -null	12 ± 3	14 ± 3	+	+
K5-Cre-ER ^{T(tg/0)} /RXR α ^{L2/L2} /RAR γ ^{-/-}	RA	RXR α -null in all layers and RAR γ -null (RXR α ^{ep-/-} /RAR γ ^{-/-})	7 ± 2	ND	+	+
CMV-Cre-ER ^{T(tg/0)} /RAR γ ^{L3/L3}	RA	RAR γ -null in granular layer only (RAR γ ^{sb-/-})	ND	13 ± 4	+	+

^aWild-type-like include RXR α ^{L2/L2}; CMV-Cre-ER^{T(tg/0)}/RAR γ ^{+L3} and CMV-Cre-ER^{T(tg/0)}/RXR α ^{+L2}; +, basal level of expression; ++ to +++, increasing level of expression; ND, not determined.

analysed RA-induced epidermal hyperplasia in mice carrying RAR- and RXR-null mutations selectively in their epidermis.

Genetic evidence that RXR α /RAR γ heterodimers are the functional units responsible for RA-induced epidermal hyperplasia

RXR α ^{L2/L2} mice were crossed with either RAR α ^{+/-} or RAR γ ^{+/-} mice. These animals were further mated with K5-Cre-ER^{T(tg/0)} transgenic mice (see above) to generate RXR α ^{L2/L2}, RAR α ^{-/-}, RAR γ ^{-/-}, K5-Cre-ER^{T(tg/0)}/RXR α ^{L2/L2}, K5-Cre-ER^{T(tg/0)}/RXR α ^{L2/L2}/RAR α ^{-/-} and K5-Cre-ER^{T(tg/0)}/RXR α ^{L2/L2}/RAR γ ^{-/-} animals, which were treated with TAM at 14 weeks of age (Figure 3A). The efficacy of RXR α gene excision in keratinocytes was quantified after separation of epidermis from dermis. Whereas no RXR α L⁻ excised allele was found in epidermis before TAM administration (Figure 3B, left panel), this treatment resulted in almost 100% L⁻ alleles in mice carrying the K5-Cre-ER^T transgene (yielding RXR α ^{ep-/-} mice), but had no effect in those lacking the transgene (Figure 3B, right panel). The RXR α L⁻ allele was never detected in the dermis (data not shown). No RXR α protein was detected by immunohistochemistry in the interfollicular epidermis and hair follicles of RXR α ^{ep-/-} mice at D11 (compare Figure 3C with D). Thus, TAM administration efficiently and selectively abolishes RXR α expression in epidermis of mice bearing the K5-Cre-ER^T transgene and the RXR α L2 alleles. Note

that the RXR α -ablated epidermis did not exhibit any interfollicular hyperplasia shortly after the TAM treatment (Figure 3D), in agreement with our previous report showing that hyperproliferation is a late event occurring 10–12 weeks after RXR α ablation (Li *et al.*, 2000).

Cell proliferation was quantified by counting BrdU-labelled basal keratinocytes (see Table I). In ‘control’ mice (RXR α ^{L2/L2}), about half (54 ± 6%) of the basal keratinocytes were BrdU positive after RA treatment (Figure 3E; note that 5 ± 2% of the basal keratinocytes were BrdU positive in vehicle-treated mice). RA-induced epidermal thickening and cell hyperproliferation in RAR α ^{-/-} mice was similar to that of controls (Figure 3F, 50 ± 4%), while it was much reduced in RAR γ ^{-/-} mice (Figure 3G, 12 ± 3%) and in RXR α ^{ep-/-} mice (Figure 3H, 12 ± 4%). Ablation of RXR α in epidermis of RAR α ^{-/-} mice (i.e. RXR α ^{ep-/-}/RAR α ^{-/-} mice) did not further decrease RA-induced proliferation (Figure 3I, 13 ± 4%). In contrast, RA treatment had almost no effect in RXR α ^{ep-/-}/RAR γ ^{-/-} mice (Figure 3J, 7 ± 2%). Thus, RXR α /RAR γ , but not RXR α /RAR α , heterodimers appear to be required within the epidermis to mediate RA-induced hyperplasia. Note that the percentage of proliferating keratinocytes in vehicle-treated mice (~6%) is below that observed in RA-treated RAR γ ^{-/-} or RXR α ^{ep-/-} epidermis (~13%), but is similar to that of RA-treated RXR α ^{ep-/-}/RAR γ ^{-/-} mice (~7%). As RAR α and RXR β are also expressed in epidermis (Fisher *et al.*, 1994; Li *et al.*, 2000), these observations suggest some functional redundancy (see

Fig. 2. Histology and proliferative response of wild-type skin upon topical retinoid treatment. Dorsal skin was treated for four consecutive days with 40 nmol (in 400 μ l acetone) of RA (B and D) or synthetic retinoids (E–J), as indicated. Controls (A and C) were treated with acetone vehicle. (A and B) Histology of control and RA-treated epidermis. Semi-thin sections (2- μ m thick) were stained with toluidine blue. Arrowheads point to the spinous and granular keratinocytes in control epidermis. (C–J) Skin sections showing the proliferation marker Ki67 (white colour) and counterstained with DAPI (blue colour). Arrows point to the dermal-epidermal junction. RA, retinoic acid; BMS493, panRAR-selective antagonist; BMS649 (SR11237), panRXR-selective agonist; BMS753, RAR α -selective agonist; BMS961, RAR γ -selective agonist. B, basal layer; C, cornified layer; D, dermis; hf, hair follicle; SB, suprabasal layers (spinous and granular keratinocytes). Scale bar: 15 μ m in (A) and (B); 25 μ m in (C)–(I).

Introduction) between RAR γ and RAR α , and between RXR α and RXR β , even though, on their own, disruption of either the RAR α gene (see above) or the RXR β gene (Kastner *et al.*, 1996) did not affect the RA-induced proliferation (data not shown). Disruption of the RAR β gene (Ghyselinck *et al.*, 1997), which is expressed in dermal fibroblasts but not in epidermis (see above), did not perturb RA-induced epidermal hyperproliferation (data not shown).

To determine in which epidermal cell layer RA exerts its primary effect (i.e. basal or suprabasal), we next ablated RAR γ and RXR α in suprabasal layers.

Genetic evidence that RAR γ is required in suprabasal cell layers for RA-induced epidermal hyperplasia

RAR $\gamma^{L3/L3}$ mice (Figure 4A; Chapellier *et al.*, 2002b) were crossed with CMV-Cre-ERT^(tg/0) transgenic mice, in which TAM administration can induce Cre-mediated recombination in suprabasal layers but not in the basal layer (Brocard *et al.*, 1997), to produce ‘control’ (CMV-Cre-ERT^(tg/0)/RAR $\gamma^{+/L3}$) and experimental (CMV-Cre-ERT^(tg/0)/RAR $\gamma^{L3/L3}$) animals. At 14 weeks of age, all mice were treated with TAM (Figure 4B) and RAR γ gene disruption was quantified. No RAR γ^L -excised allele was

Fig. 3. Conditional mutagenesis of RXR α in epidermis, and RA-induced proliferative response. (A) Scheme of the experimental protocol. Intraperitoneal injection of TAM (1 mg) was performed from day 1 (D1) to day 4 (D4). A tail biopsy was made on day 11 to check for RXR α gene disruption. RA (40 nmol) was topically applied on skin for 4 days (D12–D15). On day 16 (D16), an injection of BrdU (50 mg/kg) was given 2 h before skin sampling. (B) Efficiency of RXR α gene disruption in mice bearing the K5-Cre-ER^T transgene (as indicated). RXR α L2 and L⁻ alleles were identified on tail epidermis genomic DNA before and after TAM administration. M: DNA ladder. (C and D) Immunohistochemical detection of RXR α on skin sections from control (RXR α ^{L2/L2}) and mutant (RXR α ^{ep-/-}) mice. (E–J) Representative skin sections labelled with BrdU (brown colour), showing epidermis thickness and basal keratinocyte proliferation in mutants (genotypes as indicated). Arrows point to the dermal–epidermal junction. hf, hair follicles. Scale bar: 25 μ m in (C) and (D); 50 μ m in (E)–(J).

found before TAM treatment (Figure 4C, left panel). As expected from the selective expression of the CMV-Cre-ER^T transgene in suprabasal (sb), but not in basal keratinocytes (Brocard *et al.*, 1997), this treatment resulted only in a partial conversion of RAR γ L3 alleles into L2Neo (in which the neo gene was not removed; see Figure 4A) and L⁻ alleles (Figure 4C, middle panel). Importantly, no RAR γ L⁻ and L2Neo alleles could be detected a few weeks after the end of TAM treatment (data not shown), proving that no disruption of the RAR γ gene had occurred in basal keratinocytes (see also below; Figure 4H). Thus, TAM administration to CMV-Cre-ER^T(tg0)/RAR γ ^{L3/L3} mice

effectively resulted in selective disruption of the RAR γ gene in epidermal suprabasal layers, yielding RAR γ ^{sb-/-} mice.

In RA-treated ‘control’ mice, the epidermis was markedly thickened and about half ($56 \pm 5\%$) of basal keratinocytes expressed Ki67 (Figure 4D), instead of $6 \pm 2\%$ in vehicle-treated mice (Figure 2C; Table I). In contrast, epidermal proliferation was lower in RA-treated RAR γ ^{sb-/-} mice (Figure 4E, $13 \pm 4\%$; Table I), a situation similar to that observed in RAR γ ^{-/-} mice (Figure 4F, $14 \pm 3\%$; Table I). Following RA treatment, the RAR γ protein was easily detected by immunohistochemistry

Fig. 4. Conditional mutagenesis of *RARγ* and RA-induced proliferative response in mice lacking *RARγ* in suprabasal layers (*RARγ*^{sb-/-} mice). (A) Schematic drawing of *RARγ* wild-type (+), L3, partially excised L2Neo and fully excised L⁻ alleles. Sizes of *NsiI* fragments obtained for each allele are in kilobases (kb). Black boxes, exons 7–13 (E7–13); neo, neomycin gene; H, *HpaI*; N, *NsiI*. Arrowhead flags represent loxP sites. (B) Experimental protocol. Injection of TAM (1 mg) was performed from day 1 (D1) to day 3 (D3), then every second day for 10 days. A tail biopsy was made on day 9 to check for *RARγ* gene disruption. RA (40 nmol) was then applied topically on skin for 4 days (D10–D13). On day 14 (D14), the skin was sampled. (C) Efficiency of *RARγ* gene disruption in mice bearing the CMV-Cre-ER^T transgene. *RARγ* wild-type (+), L3, L2Neo and L⁻ alleles were detected on tail epidermis and dermis genomic DNA before and after TAM administration. Note that the minute amount of *RARγ* excised L⁻ allele present in dermis most probably originates from contaminating epidermal keratinocytes (Li *et al.*, 2000). M: DNA ladder. (D–F) Representative skin sections showing RA-induced basal keratinocyte proliferation (white colour, Ki67 signal; blue colour, DAPI nuclear staining). RA-induced epidermal proliferation in *RARγ*^{-/-} mice is illustrated in (F). (G–I) Immunohistochemical detection of *RARγ* on back skin sections from control [(G), *RARγ*^{sb+/+}] and mutant [(H), *RARγ*^{sb-/-}] mice, after RA treatment. An *RARγ*^{-/-} skin sample was used as negative control (I). Arrows point to the dermal-epidermal junction. hf, hair follicles. Scale bars (in F and I): 50 μm.

throughout the hyperplastic epidermal layers and in hair follicles of ‘control’ mice (Figure 4G). As expected, *RARγ* was also detected in basal and hair follicle keratinocytes of

RARγ^{sb-/-} mice, but not in suprabasal keratinocytes (Figure 4H; data not shown). It was also not detected in epidermis of RA-treated *RARγ*^{-/-} mice (Figure 4I).

Fig. 5. Conditional mutagenesis of RXRα in epidermis suprabasal layers, and RA-induced proliferative response in RXRα^{sb/-} mice. (A) Efficiency of RXRα gene disruption in mice bearing the CMV-Cre-ER^T transgene as compared with the K5-Cre-ER^T transgene (as indicated). The experimental protocol is the same as in Figure 4B. RXRα wild-type (+), L2 and L⁻ alleles were detected on tail epidermis genomic DNA before and after TAM administration. M: DNA ladder. (B and C) Representative back skin sections labelled with BrdU (brown colour), showing RA-induced epidermal thickening and increased basal keratinocytes proliferation. (D and E) Immuno-histochemical detection of RXRα on skin sections from control [(D), RXRα^{sb/+}] and experimental [(E), RXRα^{sb/-}] mice, after RA treatment. Arrowheads indicate nuclei of suprabasal keratinocytes expressing RXRα. Arrows point to the dermal-epidermal junction. hf, hair follicles. Scale bar (in D): 50 μm.

Importantly, RA-induced epidermal hyperproliferation in RARγ^{sb/-} mice was restored to 'control' levels a few weeks after the end of TAM treatment (data not shown), clearly demonstrating that RARγ disruption was restricted to suprabasal cells. Altogether, these results indicate that RARγ expressed in suprabasal cells is required to transduce the retinoid signal that triggers proliferation of basal keratinocytes.

Persistence of the RXRα protein in epidermal suprabasal cells in which the RXRα gene is disrupted

To determine whether RXRα could be the heterodimeric partner of RARγ in suprabasal cells, RXRα^{L2/L2} mice were crossed with CMV-Cre-ER^{T(tg/0)} transgenic mice, to produce 'control' (CMV-Cre-ER^{T(tg/0)}/RXRα^{+/L2}) and 'experimental' (CMV-Cre-ER^{T(tg/0)}/RXRα^{L2/L2}) mice. Before TAM administration, no RXRα L⁻ allele

was found in epidermis (Figure 5A, left panel), whereas this treatment induced conversion of RXRα L2 alleles into L⁻ alleles in 'experimental' mice (Figure 5A, right panel). The remaining L2 alleles originated from the basal keratinocytes, as this species was absent in DNA from an RXRα^{ep/-} mouse skin sample. Importantly, as in the case for the RARγ^{L3/L3} gene (see above), no disrupted RXRα^{L2/L2} gene could be detected a few weeks after the end of TAM treatment (data not shown), indicating that the RXRα gene was not disrupted in the basal cell layer.

Thus, TAM administration successfully induced disruption of the floxed RXRα gene in suprabasal, but not basal, keratinocytes of mice bearing the CMV-CreER^T transgene, yielding RXRα^{sb/-} mice. However, RA-induced proliferation was similar in TAM-treated 'control' and 'experimental' (RXRα^{sb/-}) mice (Figure 5B and C, 54 ± 6% and 47 ± 8%, respectively), and unexpectedly RXRα was readily detected by immunohistochemistry throughout epidermis, including in suprabasal keratinocytes (arrowheads in Figure 5E) of both RA-treated 'control' and RXRα^{sb/-} mice (compare Figure 5D with E). Thus, the RXRα protein was still present in epidermis suprabasal cells of experimental mice, even though TAM administration efficiently induced Cre-mediated disruption of the RXRα L2 alleles in these cells. This indicates that there is little turnover of the RXRα protein upon differentiation of basal into suprabasal cells.

RA-induced epidermal hyperplasia is associated with induction of HB-EGF expression

Altogether, the above observations suggest that disruption of RARγ in suprabasal keratinocytes impairs a paracrine signalling system mediating the effects of RA on basal keratinocyte proliferation. Epidermal growth is modulated by several factors, including epidermal growth factor (EGF), TGFα, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, epiregulin, heregulin and β-cellulin (Jost *et al.*, 2000). Expression of HB-EGF was previously shown to be selectively induced by RA in epidermis suprabasal layers (Stoll and Elder, 1998; Xiao *et al.*, 1999). To investigate whether the epidermal response to synthetic retinoids (see Figure 2) could be correlated with activation of the HB-EGF signalling pathway, HB-EGF mRNA levels were determined (Figure 6A). The panRAR antagonist BMS493 abolished the RA-induced increase of HB-EGF mRNA, while the increase observed upon treatment with the RARγ agonist, BMS961, was potentiated by co-administration of the panRXR agonist BMS649, which, on its own, did not induce HB-EGF mRNA expression. Thus, retinoid-induced epidermis hyperplasia is correlated with an increased expression of HB-EGF mRNA in the epidermis. Furthermore, the ability of RA to induce HB-EGF mRNA was severely impaired whenever RARγ expression was abrogated either in epidermis or selectively in suprabasal keratinocytes (i.e. in RARγ^{-/-}, RXRα^{ep/-}/RARγ^{-/-} and RARγ^{sb/-} mice), but was unaffected in mice lacking RARα (Figure 6B). Expression of HB-EGF mRNA was also significantly reduced, but not abolished, in mice lacking RXRα in epidermis (RXRα^{ep/-} animals), most probably due to some functional redundancy between RXRα and RXRβ, even though no decrease of HB-EGF transcripts was observed in the skin of RXRβ^{-/-} mice (data

Fig. 6. Expression of HB-EGF, CRABP II, RAR β 2 and β -actin mRNA in whole back skin following topical application of retinoids. (A) Northern blot analysis of total RNA (25 μ g) before (control) and after retinoid administration, as indicated. RA, retinoic acid; BMS493, panRAR antagonist; BMS961, RAR γ agonist; BMS649 (SR11237), panRXR agonist. (B) Northern blot analysis of total RNA (25 μ g) from control (RXR $\alpha^{L2/L2}$) and mutant mice (genotypes as indicated) before (-) and after (+) RA administration.

not shown). In contrast, selective disruption of the RXR α gene in suprabasal keratinocytes (RXR $\alpha^{sb-/-}$ mice) had no effect on HB-EGF mRNA levels, in keeping with persistence of the RXR α protein (see above).

The cellular retinoic acid-binding protein II (CRABP II), which is expressed in suprabasal keratinocytes, is considered as an indicator of RA activity in the epidermis (Fisher and Voorhees, 1996). Variations in levels of CRABP II mRNA in response to retinoid treatments and in RAR γ and RXR α mutants strictly paralleled those of HB-EGF mRNA (Figure 6). It is therefore likely that, within the RAR/RXR heterodimers controlling RA target gene expression in suprabasal cells (e.g. HB-EGF and CRABP II), RAR γ plays a crucial role, whereas RXR α can, to some extent, be functionally replaced by RXR β .

Interestingly, RAR β 2 mRNA, which is not or barely detectable in skin under normal conditions (Fisher *et al.*, 1994; see Figure 6B, lanes 1 and 2), but is known to be RA-inducible in dermal fibroblasts (Redfern and Todd, 1992; Tsou *et al.*, 1994), was present in all RA-treated

samples (Figure 6B), except in those from mice harbouring a germ-line RAR γ -null mutation (RAR $\gamma^{-/-}$ and RXR $\alpha^{ep-/-}$ /RAR $\gamma^{-/-}$ mice). This observation suggests that (i) topical RA treatment was efficient down to the dermis and (ii) induction of RAR β 2 expression by RA in the dermis is mediated through heterodimers involving RAR γ .

Discussion

Based on their expression patterns (Fisher and Voorhees, 1996) and on results from overexpression of dn retinoid receptors in epidermis of transgenic mice (see Introduction), it has been proposed that RARs and RXRs (notably RAR γ and RXR α) play important physiological functions in epidermis. To gain insight into the role of retinoid receptors, and more generally of RA signalling in epidermis, we have investigated here the phenotype of mutant mice in which RAR α , RAR γ and/or RXR α have been selectively disrupted in this tissue.

RARs are dispensable for homeostatic self-renewal of epidermal keratinocytes

The skin of RAR α -null and RAR β -null mutants appears normal, while skin of RAR γ -null mutants exhibit only minor defects of granular keratinocyte differentiation (Lohnes *et al.*, 1993; Lufkin *et al.*, 1993; Ghyselinck *et al.*, 1997; our unpublished results). Functional redundancy between RAR α and RAR γ (see Introduction), which, in contrast to RAR β , are both expressed in epidermis (Fisher *et al.*, 1994), could have accounted for the paucity of alterations in epidermis of RAR α - and RAR γ -null mice. Such a functional redundancy could not be assessed from examination of RAR α /RAR γ -null mice, as they die before the onset of epidermis formation (Wendling *et al.*, 2001). The inducible conditional somatic mutagenesis strategy used here has allowed us to generate mice selectively lacking both RAR α and RAR γ in adult epidermis. As RAR β is not ectopically expressed in the epidermis of these mice, they actually display a 'panRAR-null' epidermis fully devoid of RAR α , β and γ . As this panRAR-null epidermis is similar to that of a RAR γ -null mice (our unpublished results), we conclude that there is little (and possibly no) redundancy between RAR α and RAR γ for adult keratinocyte proliferation and differentiation. Moreover, as the complete absence of RARs in basal keratinocytes does not alter their homeostatic proliferation, our study demonstrates for the first time that self-renewal of epidermal keratinocytes does not require an epidermal RAR-dependent signalling pathway.

We have previously shown that RXR α ablation in basal keratinocytes results notably in hair follicle degeneration and hyperproliferation of interfollicular keratinocytes (Li *et al.*, 2000, 2001). These features most probably reflect a role of RXR α /VDR heterodimers in hair follicle cycling, and the involvement of other heterodimeric partners of RXR α in the control of interfollicular keratinocytes proliferation (Li *et al.*, 2000, 2001). Such possible partners known to be expressed in epidermis include RARs (Fisher *et al.*, 1994), TRs (Billoni *et al.*, 2000) and PPARs (Peters *et al.*, 2000; Michalik *et al.*, 2001). As RAR γ -null and RAR α /RAR γ -null keratinocytes cultured *in vitro* have been shown to be refractory to RA-induced growth arrest (Goyette *et al.*, 2000), RARs could have been the partners

of RXR α that mediate an anti-proliferative effect in resting (i.e. unstimulated) epidermis. However, our present data demonstrate that ablation of the RARs in keratinocytes does not result in epidermal hyperplasia, ruling out the possibility that RAR/RXR α heterodimers could be critically involved in controlling the keratinocyte proliferation under physiological conditions *in vivo*. Interestingly, mutant mice expressing an RXR α lacking its AF-2 ligand-dependent activation function do not exhibit an epidermal interfollicular hyperplasia (B.Mascrez, N.B.Ghyselinck, M.Mark, D.Metzger, M.Li and P.Chambon, unpublished observations). Therefore, basal keratinocyte homeostatic proliferation might be controlled through heterodimers comprising a transcriptionally inactive (unliganded) RXR α and a nuclear receptor other than a RAR.

As essential components of the vitamin A metabolic machinery are expressed in keratinocytes (Niederreither *et al.*, 2002), RA could be synthesized in the epidermis. However, the observations that (i) a RXR-specific agonist has no effect on its own on keratinocyte proliferation (Thacher *et al.*, 1997; our present data), and (ii) the panRAR antagonist BMS493 does not reduce the homeostatic rate of basal keratinocyte proliferation (data not shown), indicate that endogenous RA available for activation of RARs is either lacking, or present at very low levels in resting epidermis. Along the same lines, it is noteworthy that RA is undetectable in human epidermis (Vahlquist, 1982).

The RA signal inducing epidermal hyperproliferation is transduced through RAR γ /RXR α heterodimers in suprabasal keratinocytes

Although RARs are apparently not involved in the control of homeostatic epidermal proliferation under resting conditions, our pharmacological and genetic data demonstrate that topical treatment of skin with retinoids results in a marked increase in keratinocyte proliferation that is mediated in suprabasal keratinocytes through RAR γ /RXR heterodimers. In human and mouse epidermis, RAR γ represents 90% of the RARs, the remaining 10% being RAR α (Fisher *et al.*, 1994; our unpublished results). The fact that RAR γ -null epidermis exhibits some RA-induced increased proliferation raised the possibility that RAR α could be involved in a RA-triggered cell proliferation pathway distinct from that involving RAR γ . However, RA-induced hyperproliferation of RAR α -null epidermis is identical to that of wild-type epidermis, and hyperproliferation is not induced by a RAR α -selective agonist. Thus, the residual hyperproliferation observed in RAR γ -null epidermis probably results from a functional redundancy with RAR α , which is artefactually generated by the knockout of RAR γ and similar to that previously seen in RAR γ -null cells *in vitro* (Taneja *et al.*, 1996; see Introduction for further references). Similarly, the slight increase in proliferation still observed upon RA-stimulation of epidermis lacking RAR γ selectively in the suprabasal layers (RAR $\gamma^{sb-/-}$ mice; Table I) could also reflect a redundancy with RAR α . Therefore, RAR γ is the main, if not the only, RAR-mediating RA-induced keratinocyte hyperproliferation in wild-type mice.

Likewise, the residual RA-induced proliferation increase in RXR α -null epidermis most probably reflects a functional redundancy with RXR β (Li *et al.*, 2000).

In any event, the striking synergism between RAR γ - and RXR α -null mutations strongly supports the conclusion that RXR α /RAR γ heterodimers are the functional units mediating RA-induced epidermal hyperplasia. The alternative possibility that two different converging pathways could be involved in the generation of keratinocyte hyperproliferation, the first one requiring RAR γ and the other one RXR α , is unlikely: (i) the RXR-specific agonist BMS649 has no effect on its own, indicating that RA acts neither through RXR homodimers nor through heterodimers in which RXR activity is not subordinated to that of its partner (e.g. RXR/PPAR heterodimers); and (ii) the effect of the RAR γ -selective agonist is potentiated by the RXR-selective agonist. It is noteworthy that our present study also demonstrates that the subordination of the ligand-dependent RXR activity to its liganded-RAR partner that was previously evidenced *in vitro* (Roy *et al.*, 1995; Taneja *et al.*, 1996), also occurs in the animal.

Paradoxically, RA induces opposite responses in keratinocytes depending on whether they are studied *in vivo* or cultured *in vitro*. For example, RA treatment induces keratinocyte growth-arrest *in vitro* (Goyette *et al.*, 2000) and has no effect on CRABP II expression (Fisher and Voorhees, 1996). In contrast, RA stimulates keratinocyte proliferation and increases transcription of the CRABP II gene *in vivo*. We have shown here that these RA effects are transduced by RAR γ present in epidermis suprabasal cell layers. Thus, the failure of RA to increase proliferation and CRABP II expression in cultured keratinocytes may just reflect their similarity with basal keratinocytes. This raises the interesting possibility that the RAR γ -dependent pathway involved in inhibition of keratinocyte proliferation *in vitro* (Goyette *et al.*, 2000) may also operate in basal keratinocytes *in vivo*, in order to finely tune pathophysiological epidermal responses to RA signalling, for example during skin wound healing (see below).

The RA signal transduced by RAR γ /RXR α heterodimers in suprabasal keratinocytes generates a paracrine signal inducing hyperproliferation of basal keratinocytes

As cell proliferation only takes place in the basal layer, our finding that RAR γ /RXR α heterodimers present in suprabasal keratinocytes are required for RA-induced epidermal hyperplasia demonstrates that retinoids induce, through these heterodimers, the synthesis of a paracrine signal in suprabasal keratinocytes, which in turn causes hyperproliferation of basal keratinocytes. This is in keeping with previous data showing that expression of a dnRAR α in suprabasal layers abrogate RA-induced hyperplasia (Xiao *et al.*, 1999). However, as an excess of dnRAR may, through binding to RXRs, interfere with the function of both RARs and a number of other heterodimeric partners of RXRs (e.g. PPARs), this previous study could not reveal which RXR/NR heterodimer generates the RA-induced hyperproliferation signal in suprabasal cells.

Several growth factors released from both the epidermis and the dermis affect proliferation of basal keratinocytes through binding to epidermal growth factor receptors that

are located in basal keratinocyte membranes and belong to the ErbB family (Piepkorn *et al.*, 1998; Jost *et al.*, 2000; Werner and Smola, 2001). Amongst the ligands for the ErbB isoforms that are expressed in epidermis, HB-EGF appears to be the only one regulated by RA (Stoll and Elder, 1998; Xiao *et al.*, 1999). Furthermore, its mRNA is exclusively expressed in suprabasal keratinocytes (Xiao *et al.*, 1999), whereas mature HB-EGF proteins are localized only in epidermal basal cells (Downing *et al.*, 1997). Our present data demonstrate that RXR α /RAR γ heterodimers play a key role in RA-induced HB-EGF expression in suprabasal cells, although they do not reveal whether these heterodimers act directly, or rather through a RA-responsive factor, on this expression. Our study also underlines a positive correlation between expression of HB-EGF and RA-induced proliferation (Table I), further supporting the possibility that HB-EGF could be 'the' paracrine factor that is synthesized in the suprabasal layers and mediates RA-induced hyperplasia (Xiao *et al.*, 1999). We note, however, that RA does not increase HB-EGF expression in RAR $\gamma^{-/-}$ and RAR $\gamma^{sb/-}$ mice, whereas it still exerts some proliferative effect on their epidermis (Table I). Thus, HB-EGF may not be the only signalling molecule involved in RA-induced proliferation. Interestingly, a recent study has shown that expression of keratinocyte growth factor is stimulated by RA in cultured gingival fibroblasts (Mackenzie and Gao, 2001).

In conclusion, our data indicate that a retinoid-inducible RAR γ /RXR α heterodimer-mediated pathway, which functions in epidermal suprabasal cells to synthesize a paracrine signal triggering hyperproliferation of basal keratinocytes, is dispensable for their homeostatic proliferation in resting skin. It is tempting to speculate that this pathway could be required under stress conditions, such as wound healing. In this respect, it is noteworthy that: (i) vitamin A deficiency causes delayed wound healing, whereas pre-treatment with vitamin A or RA improves epidermal regeneration (Hunt, 1986); (ii) HB-EGF secretion is increased in response to epidermal injury (Marikovsky *et al.*, 1993; McCarthy *et al.*, 1996); (iii) RA-induced HB-EGF expression occurs prior to the onset of basal cell layer proliferation (Xiao *et al.*, 1999). Thus, the possibility exists that the beneficial actions of retinoids during wound healing could be mediated through activation of RAR γ /RXR α heterodimers, which at the very least may control HB-EGF signalling in suprabasal keratinocytes.

Materials and methods

Mice and treatments

RAR α - and RAR γ -null, floxed RXR α , RAR α and RAR γ , and CMV-Cre-ERT⁺ and K5-Cre-ERT⁺ transgenic lines have been described previously (Lohnes *et al.*, 1993; Lufkin *et al.*, 1993; Brocard *et al.*, 1997; Indra *et al.*, 1999; Li *et al.*, 2000; Chapellier *et al.*, 2002a,b). Tail epidermis was separated from dermis as described previously (Li *et al.*, 2000). Each experiment (two to three females of each genotype) was repeated three times. Neither acetone nor TAM treatments affected epidermal proliferation.

Histochemistry and proliferation analysis

For histology, skin samples were fixed with 2.5% glutaraldehyde (0.1 M cacodylate pH 7.2). RXR α and RAR γ immunolocalization were as described previously (Ghyselinck *et al.*, 1997; Li *et al.*, 2001). Proliferating cells were detected using an anti-BrdU antibody, revealed

by peroxidase activity and counterstained with Harris haematoxylin. The proliferation marker Ki67 was detected by immunohistochemistry (Novocastra's protocol) on frozen sections post-fixed in 2% paraformaldehyde, and counterstained with 0.01% DAPI. The number of either BrdU- or Ki67-positive and total basal keratinocytes were counted on five areas from three animals of each genotype. The mean $\pm$ SD percentage of proliferating over total basal cells (>200) was then estimated.

Supplementary data

More details of Materials and methods are available as supplementary data at *The EMBO Journal* Online, or on request.

Acknowledgements

We are grateful to C.Suzi (Bristol-Myers Squibb) for synthetic retinoids. We thank B.Féret, B.Bondeau, I.Tilly, B.Weber, O.Wendling and C.Dennefeld for excellent technical assistance, as well as the staff of the animal facility. We are indebted to A.Dierich and E.Blondelle for embryonic stem cell culture. This work was supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Collège de France, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Human Frontier Science Program, the Ministère de l'Éducation Nationale de la Recherche et de la Technologie and the EEC (CT 97-3220).

References

- Billoni, N., Buan, B., Gautier, B., Gaillard, O., Mahé, Y.F. and Bernard, B.A. (2000) Thyroid hormone receptor β 1 is expressed in the human hair follicle. *Br. J. Dermatol.*, **142**, 645–652.
- Brocard, J., Warot, X., Wendling, O., Messaddeq, N., Vonesch, J.L., Chambon, P. and Metzger, D. (1997) Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc. Natl Acad. Sci. USA*, **94**, 14559–14563.
- Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J.*, **10**, 940–954.
- Chapellier, B., Mark, M., Garnier, J.M., LeMour, M., Chambon, P. and Ghyselinck, N.B. (2002a) A conditional floxed (loxP-flanked) allele for the Retinoic Acid Receptor alpha (RAR α) gene. *Genesis*, **32**, 87–89.
- Chapellier, B., Mark, M., Garnier, J.M., Dierich, A., Chambon, P. and Ghyselinck, N.B. (2002b) A conditional floxed (loxP-flanked) allele for the Retinoic Acid Receptor gamma (RAR γ) gene. *Genesis*, **32**, 95–98.
- Chen, J.D. and Evans, R.M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*, **377**, 454–457.
- Chen, S. *et al.* (1995) Retinoic acid receptor γ mediates topical retinoid efficacy and irritation in animal models. *J. Invest. Dermatol.*, **104**, 779–783.
- Downing, M.T., Brigstock, D.R., Luquette, M.H., Crissman-Combs, M. and Besner, G.E. (1997) Immunohistochemical localization of heparin-binding epidermal growth factor-like growth factor in normal skin and skin cancers. *Histochem. J.*, **29**, 735–744.
- Elias, P.M., Fritsch, P.O., Lampe, M., Williams, M.L., Brown, B.E., Nemanic, M. and Grayson, S. (1981) Retinoid effects on epidermal structure, differentiation and permeability. *Lab. Invest.*, **44**, 531–540.
- Engelke, M., Jensen, J.M., Ekanayake-Mudiyanse, S. and Proksch, E. (1997) Effects of xerosis and ageing on epidermal proliferation and differentiation. *Br. J. Dermatol.*, **137**, 219–225.
- Feng, X., Peng, Z.H., Di, W., Li, X.Y., Rochette-Egly, C., Chambon, P., Voorhees, J.J. and Xiao, J.H. (1997) Suprabasal expression of a dominant-negative RXR α mutant in transgenic mouse epidermis impairs regulation of gene transcription and basal keratinocyte proliferation by RAR-selective retinoids. *Genes Dev.*, **11**, 59–71.
- Fisher, G.J. and Voorhees, J.J. (1996) Molecular mechanisms of retinoid actions in skin. *FASEB J.*, **10**, 1002–1013.
- Fisher, G.J., Talwar, H.S., Xiao, J.H., Datta, S.C., Reddy, A.P., Gaub, M.P., Rochette-Egly, C., Chambon, P. and Voorhees, J.J. (1994) Immunological identification and functional quantitation of retinoic acid and retinoid X receptor proteins in human skin. *J. Biol. Chem.*, **269**, 20629–20635.
- Frazier, C.N. and Hu, C.K. (1931) Cutaneous lesions associated with a deficiency in vitamin A in man. *Arch. Intern. Med.*, **48**, 507–514.
- Ghyselinck, N.B., Dupé, V., Dierich, A., Messaddeq, N., Garnier, J.M., Rochette-Egly, C., Chambon, P. and Mark, M. (1997) Role of the

- retinoic acid receptor beta (RAR β) during mouse development. *Int. J. Dev. Biol.*, **41**, 425–447.
- Goyette,P., Feng-Chen,C., Wang,W., Seguin,F. and Lohnes,D. (2000) Characterization of retinoic acid receptor-deficient keratinocytes. *J. Biol. Chem.*, **275**, 16497–16505.
- Hunt,T.K. (1986) Vitamin A and wound healing. *J. Am. Acad. Dermatol.*, **15**, 817–821.
- Imakado,S. *et al.* (1995) Targeting expression of a dominant-negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function. *Genes Dev.*, **9**, 317–329.
- Indra,A.K., Warot,X., Brocard,J., Bornert,J.M., Xiao,J.H., Chambon,P. and Metzger,D. (1999) Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER^T and Cre-ER^{T2} recombinases. *Nucleic Acids Res.*, **27**, 4324–4327.
- Jost,M., Kari,C. and Rodeck,U. (2000) The EGF-receptor—an essential regulator of multiple epidermal functions. *Eur. J. Dermatol.*, **10**, 505–510.
- Kastner,P., Mark,M. and Chambon,P. (1995) Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell*, **83**, 859–869.
- Kastner,P. *et al.* (1996) Abnormal spermatogenesis in RXR β mutant mice. *Genes Dev.*, **10**, 80–92.
- Kastner,P., Mark,M., Ghyselinck,N., Krezel,W., Dupé,V., Grondola,J.M. and Chambon,P. (1997) Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. *Development*, **124**, 313–326.
- Li,M., Indra,A.K., Warot,X., Brocard,J., Messaddeq,N., Kato,S., Metzger,D. and Chambon,P. (2000) Skin abnormalities generated by temporally controlled RXR α mutations in mouse epidermis. *Nature*, **407**, 633–636.
- Li,M., Chiba,H., Warot,X., Messaddeq,N., Gérard,C., Chambon,P. and Metzger,D. (2001) RXR α ablation in skin keratinocytes results in alopecia and epidermal alterations. *Development*, **128**, 675–688.
- Livrea,M.A. (2000) *Vitamin A and Retinoids: An Update of Biological Aspects and Clinical Applications*. Birkhauser Verlag, Basel, Switzerland.
- Lohnes,D., Kastner,P., Dierich,A., Mark,M., LeMeur,M. and Chambon,P. (1993) Function of retinoic acid receptor γ in the mouse. *Cell*, **73**, 643–658.
- Lufkin,T., Lohnes,D., Mark,M., Dierich,A., Gorry,P., Gaub,M.P., LeMeur,M. and Chambon,P. (1993) High postnatal lethality and testis degeneration in retinoic acid receptor α mutant mice. *Proc. Natl Acad. Sci. USA*, **90**, 7225–7229.
- Mackenzie,I.C. and Gao,Z. (2001) Keratinocyte growth factor expression in human gingival fibroblasts and stimulation of *in vitro* gene expression by retinoic acid. *J. Periodontol.*, **72**, 445–453.
- Mangelsdorf,D.J. *et al.* (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.
- Marikovsky,M., Breuing,K., Liu,P.Y., Eriksson,E., Higashiyama,S., Farber,P., Abraham,J. and Klagsbrun,M. (1993) Appearance of heparin-binding EGF-like growth factor in wound fluid as a response to injury. *Proc. Natl Acad. Sci. USA*, **90**, 3889–3893.
- Mascrez,B., Mark,M., Dierich,A., Ghyselinck,N.B., Kastner,P. and Chambon,P. (1998) The RXR α ligand-dependent activation function 2 (AF-2) is important for mouse development. *Development*, **125**, 4691–4707.
- McCarthy,D.W., Downing,M.T., Brigstock,D.R., Luquette,M.H., Brown,K.D., Abad,M.S. and Besner,G.E. (1996) Production of heparin-binding epidermal growth factor-like growth factor (HB-EGF) at sites of thermal injury in pediatric patients. *J. Invest. Dermatol.*, **106**, 49–56.
- Metzger,D. and Chambon,P. (2001) Site- and time-specific gene targeting in the mouse. *Methods*, **24**, 71–80.
- Michalik,L. *et al.* (2001) Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR) α and PPAR β mutant mice. *J. Cell Biol.*, **154**, 799–814.
- Morris-Kay,G.M. and Ward,S.J. (1999) Retinoids and mammalian development. *Int. Rev. Cytol.*, **188**, 73–131.
- Niederreither,K., Fraulob,V., Garnier,J.M., Chambon,P. and Dollé,P. (2002) Differential expression of retinoic-acid-synthesizing (RALDH) enzymes during fetal development and organ differentiation in the mouse. *Mech. Dev.*, **110**, 165–171.
- Peters,J.M., Lee,S.S., Li,W., Ward,J.M., Gavrilova,O., Everett,C., Reitman,M.L., Hudson,L.D. and Gonzalez,F.J. (2000) Growth, adipose, brain and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β (δ). *Mol. Cell Biol.*, **20**, 5119–5128.
- Piepkorn,M., Pittelkow,M.R. and Cook,P.W. (1998) Autocrine regulation of keratinocytes: the emerging role of heparin-binding, epidermal growth factor-related growth factors. *J. Invest. Dermatol.*, **111**, 715–721.
- Redfern,C.P.F. and Todd,C. (1992) Retinoic acid receptor expression in human skin keratinocytes and dermal fibroblast *in vitro*. *J. Cell Sci.*, **102**, 113–121.
- Roy,B., Taneja,R. and Chambon,P. (1995) Synergistic activation of retinoic acid (RA)-responsive genes and induction of embryonal carcinoma cell differentiation by an RA receptor alpha (RAR α), RAR β , or RAR γ -selective ligand in combination with a retinoid X receptor-specific ligand. *Mol. Cell Biol.*, **15**, 6481–6487.
- Saitou,M., Sugai,S., Tanaka,T., Shimouchi,K., Fuchs,E., Narumiya,S. and Kakizuka,A. (1995) Inhibition of skin development by targeted expression of a dominant negative retinoic acid receptor. *Nature*, **374**, 159–162.
- Stoll,S.W. and Elder,J.T. (1998) Retinoid regulation of heparin-binding EGF-like growth factor gene expression in human keratinocytes and skin. *Exp. Dermatol.*, **7**, 391–397.
- Taneja,R., Roy,B., Plassat,J.L., Zusi,C.F., Ostrowski,J., Reczek,P.R. and Chambon,P. (1996) Cell-type and promoter-context dependent retinoic acid receptor (RAR) redundancies for RAR β 2 and Hoxa-1 activation in F9 and P19 cells can be artefactually generated by gene knockouts. *Proc. Natl Acad. Sci. USA*, **93**, 6197–6202.
- Thacher,S.M., Standeven,A.M., Athanikar,J., Kopper,S., Castilleja,O., Escobar,M., Beard,R.L. and Chandraratna,R.A. (1997) Receptor specificity of retinoid-induced epidermal hyperplasia: effect of RXR-selective agonists and correlation with topical irritation. *J. Pharmacol. Exp. Ther.*, **282**, 528–534.
- Tsou,H.C., Lee,X., Si,S.P. and Peacocke,M. (1994) Regulation of retinoic acid receptor expression in dermal fibroblasts. *Exp. Cell Res.*, **211**, 74–81.
- Vahlquist,A. (1982) Vitamin A in human skin: I. detection and identification of retinoids in normal epidermis. *J. Invest. Dermatol.*, **79**, 89–93.
- Wendling,O., Ghyselinck,N.B., Chambon,P. and Mark,M. (2001) Roles of retinoic acid receptors in early embryonic morphogenesis and hindbrain patterning. *Development*, **128**, 2031–2038.
- Werner,S. and Smola,H. (2001) Paracrine regulation of keratinocyte proliferation and differentiation. *Trends Cell Biol.*, **11**, 143–146.
- Wolbach,S.B. and Howe,P.R. (1925) Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.*, **42**, 753–777.
- Xiao,J.H., Feng,X., Di,W., Peng,Z.H., Li,L.A., Chambon,P. and Voorhees,J.J. (1999) Identification of heparin-binding EGF-like growth factor as a target in intercellular regulation of epidermal basal cell growth by suprabasal retinoic acid receptors. *EMBO J.*, **18**, 1539–1548.

Received March 18, 2002; revised April 29, 2002;
accepted May 6, 2002