Both Retinoic Acid Receptors α (RARα) and γ (RARγ) Are Able to Initiate Mouse Upper-Lip Skin Glandular Metaplasia

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Embryonic mouse upper-lip skin explants treated with 16.7 μM all-*trans* retinoic acid (*t*RA) give rise to a glandular metaplasia of hair vibrissa follicles; however, at this concentration, *t*RA can activate not only the three retinoic acid receptors (RARα, β, and γ), but also the retinoid X receptors (RXRα, β, and γ) as a consequence of its isomerization to 9-*cis* retinoic acid. We therefore studied the respective roles of the RXR and RAR by treating RARα$^{-/-}$, β$^{-/-}$, and γ$^{-/-}$ skin explants with *t*RA and wild-type explants with synthetic retinoids specific for RXR or for each of the RAR. The null mutation of the RARα, RARβ, and RARγ genes did not prevent *t*RA-induced hair glandular metaplasia, but RARγ inactivation dramatically reduced its ratio. As demonstrated by treating explants with a RAR- or a RXR-specific panagonist (CD367 and Ro25–7386, respectively), RAR are primarily responsible for this metaplasia. The use of two retinoids (Ro40–6055, 8×10^{-3} μM, or CD437, 7.7×10^{-2} μM) that are believed to act, respectively, as a RARα- or a RARγ-specific agonist showed that both these receptors can initiate a metaplasia. In contrast, BMS453, a RARβ-specific agonist, was unable to give rise to any metaplasia. Nevertheless, the highest degrees and ratios of metaplasia were only obtained after treatment with the CD367 RAR panagonist, or with either Ro40–6055 or CD437 at a concentration sufficient to allow the activation of the three RAR, suggesting that RARβ activation is required for a metaplasia of all vibrissæ. ***Key words: RAR null mutants/RAR-specific agonists/RXR-specific agonists/skin differentiation. J Invest Dermatol 111:206–212, 1998***

For more than 40 y, it has been appreciated that vitamin A is a critical regulator of growth and differentiation of developing adult mammalian and avian skin (reviewed by Means and Gudas, 1995). Vitamin A deficiency and hypervitaminosis A cause disruption of normal cellular homeostatic mechanism, resulting in impairment of skin barrier function. More recent studies have shown that all-*trans* retinoic acid (*t*RA) is the major biologic active form of vitamin A. Retinoids mediate their effects through two families of nuclear receptors, each being encoded by three genes: the retinoic acid receptors α, β, and γ (RARα, β, and γ) and the retinoid X receptors α, β, and γ (RXRα, β, and γ), (reviewed by Kastner *et al*, 1995; Mangelsdorf and Evans, 1995; Chambon, 1996). Among the naturally occurring retinoids, *t*RA activates RAR, whereas 9-*cis*-retinoic acid activates both RAR and RXR. All-*trans* RA treatment has marked effects on embryonic skin differentiation, leading to the exchange of one developmental pathway for another: in the mouse, glomerular glands (GG) develop instead of hair vibrissae in embryonic upper-lip skin (Hardy, 1968, 1983; Viallet *et al*, 1991; Viallet and Dhouailly, 1994a). This homeotic-type effect is stage specific: it occurs only if exogenous *t*RA is added when hair vibrissæ primordia are initiated (stages 1–3, as defined by Hardy, 1968) in response to the dermal induction.

In the mouse, the skin develops two types of hair follicles: hair pelage and, in the facial region, sensory hair follicles or vibrissæ, which are mainly grouped around the mouth and the eyes. With the exception of nasal and plantar sweat glands, the only glands present in mice skin are tiny sebaceous glands (SG), each of which arises as a bud from one side of the upper part of the pelage and vibrissa hair follicles. During development, the formation of the first vibrissæ starts at 12 d postcoitum, i.e., 2 d before the beginning of hair pelage formation. They form in sequence along five rows on the upper-lip skin, so that several developmental stages can be observed at the same time (**Fig 1*A***). The first step of hair vibrissa morphogenesis involves a placodal thickening of the epidermis associated to a condensation of dermal cells (stage 1). At stage 2, an epithelial column extends into the dermis. The dermal papilla remains in association with the tip of the invaginating peg, whose proximal end is first flat (stage 3a) and then concave (stages 3b, c). Following this, the inner root sheath forms (stages 4, 5), and then the hair shaft progressively develops and grows out of the skin (stages 6–8). The formation of the vibrissa-associated SG begins at stage 7. Previous studies have shown that hair follicles form as a result of reciprocal dermal–epidermal interactions (Dhouailly, 1977). It has been proposed that the RAR may play a role in the establishment of the dermal–epidermal interactions that lead to the formation of the hair follicle. More precisely, the transcription of the RARα and γ genes appears to be correlated with the manifestation of the inductive ability of the embryonic dermal papilla, whereas the RARβ gene is not transcribed at a detectable level during mouse skin morphogenesis (Viallet and Dhouailly, 1994b). Targeted disruption through homolog-

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Abbreviations: AGG, vibrissa-associated glomerular gland; GG, glomerular gland; GSG, hyperdeveloped sebaceous gland associated with a glomerular gland; pan-RAR, RAR panagonist; pan-RXR, RXR panagonist; RARα/β/γ, retinoic acid receptor α/β/γ; RXRα/β/γ, retinoid X receptor α/β/γ; SG, sebaceous gland; *t*RA, all-*trans* retinoic acid; WT, wild-type.

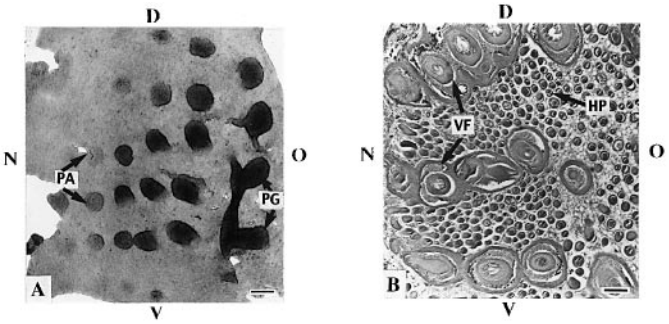

Figure 1. Distribution pattern of mouse WT upper-lip hair vibrissæ. (*A*) Isolated epidermis from a 13.5 d embryo, showing five rows of vibrissa anlagen from placodal (PA) to hair peg (PG) stages. Osmium tetroxyde fixation. *Scale bar*, 200 μm. (*B*) Tangential and partial section of a control 13.5 d explant after 48 h *in vitro* followed by 10 d onto nude mice kidney: differentiation of three rows of hair vibrissa follicles (VF), intermingled with numerous hair pelage follicles (HP). Hematoxylin/Biebrich scarlet staining. *Scale bar*, 100 μm. D/V, dorso/ventral; O/N, ocular/nasal.

ous recombination of the RARα (Lufkin *et al*, 1993) or the RARγ (Lohnes *et al*, 1993), however, as well as that of both these receptors (Lohnes *et al*, 1994), does not appear to lead to an impairment of cutaneous appendages morphogenesis. As expected, RARβ knockout also has no effect on skin development (Ghyselinck *et al*, 1997).

The treatment of these three RAR null mutants with *t*RA, by comparison with wild-type (WT) embryos, and the use of synthetic retinoids that selectively activate all RAR or RXR, or only one member of the RAR family, now give new opportunities to analyze the role of the RAR in *t*RA induced glandular metaplasia. In this study, we first checked whether the RARα, β, or γ knockout does not alter the response to the *t*RA treatment. RAR null mutants, and especially double knockout mutants, have a very reduced viability, and thus are very difficult to obtain in large numbers. Furthermore, *t*RA may possibly transactivate the RXR through isomerization (Heyman *et al*, 1992; Levin *et al*, 1992; Allegretto *et al*, 1993). We therefore used synthetic retinoids specific either for the RAR or for the RXR to discern the respective involvement of these receptors in *t*RA induced glandular metaplasia. A complementary study, using RAR agonists with different specificities, gave us a further insight about the respective ability of the RARα, β, and γ to induce a glandular metaplasia of hair vibrissa follicles.

MATERIALS AND METHODS

Obtention of embryos WT embryos were obtained by mating Swiss OF1 mice overnight. Females were examined for a vaginal plug the following morning. The day of the vaginal plug was designated as 0.5. The generation of RARα, β, or γ single null mutants has been described (Lufkin *et al*, 1993; Lohnes *et al*, 1993; Ghyselinck *et al*, 1997). Embryos were obtained by mating RARα, β, or γ appropriate heterozygotes. Genotypes were determined by genomic Southern blotting using DNA isolated from embryonic tissues. Probe, digest, and other conditions for Southern blotting have been detailed elsewhere (Lufkin *et al*, 1993; Lohnes *et al*, 1993; Ghyselinck *et al*, 1997).

***In vitro* culture and graft of skin explants** The embryonic upper-lip skin was dissected at 13.5 d of gestation. Explants were cultured *in vitro* by placing them onto a grid in a Falcon dish containing Dubelcco's modified Eagle's medium, 20% fetal calf serum, and the retinoid of interest or its solvent alone. Culture dishes were incubated for 2 d at 37°C in a 5% CO_2 atmosphere. Explants were then grafted onto nude mice kidney and allowed to develop for 10 d. After fixation in 4% formaldehyde for 12 h, the explants were dehydrated and embedded in paraffin. Transversal or sagittal sections (7 μm) were stained with Hematoxylin/Biebrich scarlet and allowed for histologic analysis.

Retinoids assays *t*RA, Ro25–7386 (RXR panagonist or pan-RXR) and Ro40–6055 (= AM580, RARα-specific agonist) were a gift from Dr. M. Klaus (Hoffman-la Roche, Basel, Switzerland); CD367 (RAR panagonist or pan-RAR) and CD437 (RARγ-specific agonist) were provided by Dr. M. Demarchez (CIRD-Galderma, Sophia Antipolis, France); BMS453 (RARβ-specific agonist) and BMS649 (another pan-RXR) were obtained from BMS Pharmaceutical Research Institute (Buffalo, NY). *t*RA was dissolved in absolute ethanol from which fresh dilution was made before use. Synthetic retinoids were dissolved in dimethylsulfoxide. In each experiment, controls were run using the same concentration of solvent (either ethanol or dimethylsulfoxide) as used in the experiment plates. Retinoids were used in the tissue culture medium at the following concentrations: *t*RA, 16.5 μM; CD367 (pan-RAR), 10^{-3}, 1, or 10 μM; Ro25–7386 (pan-RXR), 10^{-3}, 1, or 10 μM; Ro40–6055, 10^{-3}, 8×10^{-3} (concentration recommended for RARα specificity), 10^{-2}, 10^{-1}, or 1 μM; BMS453, 10^{-3}, 10^{-2}, 10^{-1} (concentration recommended for RARβ specificity), or 1 μM; CD437, 10^{-3}, 10^{-2}, 7.7×10^{-2} (concentration recommended for RARγ specificity), 10^{-1}, or 1 μM; BMS649 (pan-RXR), 10^{-1} μM. All procedures involving manipulation of retinoids were performed in dim light.

RESULTS

RARα, β, or γ null mutation does not impair the proper development of mouse upper-lip skin At 13.5 d of gestation, the morphology of the RARα$^{-/-}$, β$^{-/-}$, and γ$^{-/-}$ explants was similar to that of the WT explants: their vibrissæ were also distributed in five rows, the posterior and ventral vibrissæ, respectively, developing earlier than the anterior and dorsal ones, as shown for a WT explant (**Fig 1*A*, *B***). After 2 d *in vitro* followed by 10 d on the nude mice kidney, the appearance of the RARα$^{-/-}$, β$^{-/-}$, and γ$^{-/-}$ explants was similar to that of the WT. In particular, the morphology of the hair and vibrissa follicles was identical to that of the WT standards (**Fig 2*A*, *C*, *E*, *G***), with the usual small SG connected to the upper part of the follicles (not shown).

RARγ knockout dramatically decreases the ratio of *t*RA-induced glandular metaplasia We then checked the ability of skin explants derived from 13.5 d RARα$^{-/-}$, β$^{-/-}$, or γ$^{-/-}$ null mutant embryos to respond to the *t*RA treatment. Unexpectedly, all three kinds of explants were still able to give rise to a glandular metaplasia of vibrissæ (**Fig 2*D*, *F*, *H***), the morphology of the glands being similar to those obtained with the WT explants (**Fig 2*B***). Some vibrissæ totally disappeared and were replaced by a GG displaying a small glomerule and a duct opening directly onto the surface of the epidermis (**Fig 2*B*, *D*, *F***). Secretory elements were present in the lumen of some glomerules, suggesting that the corresponding glands were active (**Fig 2*D***). In a few cases, the glomerule did not form, and the gland was restricted to a duct that probably represents an incomplete type of metaplasia (not shown). Furthermore, some glands appeared to grow out from the outer root sheath of the hair vibrissa follicles (**Fig 2*H***). These vibrissa-associated glands (AGG) nevertheless displayed a glomerule and a duct similar to those of the GG. The localization of the duct connection was not conserved among the different follicles: some ducts grew out from the basis of the follicle whereas others grew from the upper third.

Whereas *t*RA induced a glandular metaplasia in all WT, RARα$^{-/-}$, and β$^{-/-}$ explants, only three of five treated γ$^{-/-}$ explants actually responded (**Table I**). Furthermore, RARα$^{-/-}$ and β$^{-/-}$ explants presented an identical (50%) ratio of vibrissa glandular metaplasia (calculated as the mean percentage of vibrissæ transformed into glands) (**Fig 3**): this ratio is not very different from that of the WT explants (58%). In contrast, only 20% of the vibrissæ of the responsive RARγ$^{-/-}$ explants gave rise to a glandular metaplasia. It should be noted that the total mean number of appendages (involving vibrissæ and glands) per explant after *t*RA treatment was of the same order for all four genotypes. The occurrences of the three types of metaplasia obtained with the four genotypes are summarized in **Table I**. The ratio of AGG was comparable for the three kinds of mutants, and not significantly different from the one obtained with the WT explants; however, the degree of metaplasia (i.e., the ratio of GG *versus* AGG) varies between 1 (RARγ$^{-/-}$), 1.8 (WT), 2.7 (RARα$^{-/-}$), and 3 (RARβ$^{-/-}$). The RARγ$^{-/-}$ explants thus displayed the lowest ratio as well as the lowest degree of glandular metaplasia.

Panagonist specific for RAR, but not for RXR, can transform almost all vibrissæ into glands RAR null mutants, and especially double knockout mutants, have a very reduced viability, thus impairing their use in a large series of grafting experiments. Furthermore, *t*RA may possibly transactivate the RXR through its isomerization (Heyman

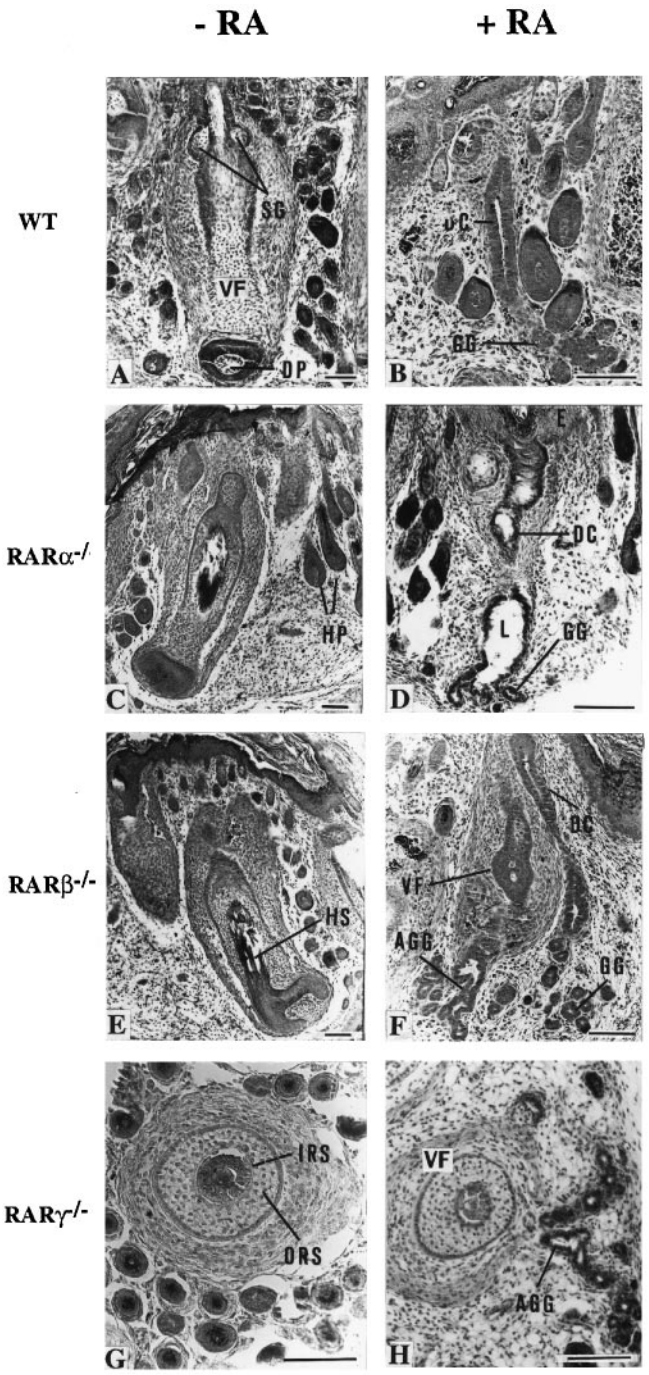

Figure 2. *t*RA induces a hair vibrissa metaplasia in mouse upper-lip skin explants from WT embryos as well as from RARα$^{-/-}$, β$^{-/-}$, or γ$^{-/-}$ null mutants. Upper-lip skin explants from 13.5 d embryos were cultured *in vitro* for 2 d in the absence (–RA) or the presence (+RA) of 16.5 μM *t*RA. They were then grafted onto nude mice kidney for 10 d. Untreated WT (*A*) as well as RARα$^{-/-}$ (*C*), RARβ$^{-/-}$ (*E*), and RARγ$^{-/-}$ (*G*) explants developed normal vibrissa and pelage hair follicles. After *t*RA treatment, similar glandular structures were observed in WT (*B*), RARα$^{-/-}$ (*D*), RARβ$^{-/-}$ (*F*), and RARγ$^{-/-}$ (*H*) explants. (*A–F*) Longitudinal sections. (*G*, *H*) Transversal sections. AGG, Glomerular gland associated with a vibrissa; DC, duct; DP, dermal papilla; E, epidermis; GG, glomerular gland; HP, hair pelage follicle; HS, hair shaft; IRS, inner root sheath; L, lumen; ORS, outer root sheath; SG, sebaceous gland; VF, hair vibrissa follicle. (*A–H*) Hematoxylin/Biebrich scarlet staining. *Scale bar*, 100 μm.

et al, 1992; Levin *et al*, 1992; Allegretto *et al*, 1993). We therefore used synthetic retinoids specific either for the RAR (the pan-RAR CD367) or for the RXR (the pan-RXR Ro25–7386) to discern the respective involvement of these two families of receptors in *t*RA induced glandular metaplasia. All explants treated with one of these two molecules gave rise to a glandular metaplasia of vibrissæ (**Table II**). Futhermore, at 10^{-3} μM, the pan-RAR resulted in a metaplasia of about 90% of the vibrissæ (**Fig 4*A***). Increasing the dose of the pan-RAR led to a complete disappearance of typical vibrissa follicles. In contrast, after a treatment with 10^{-3} μM pan-RXR, only 20% of the vibrissæ of each explant gave rise to a glandular metaplasia (**Fig 4*B***). Even at a 10,000 times higher concentration (10 μM), the Ro25–7386 pan-RXR failed to transform all the vibrissæ into glands. Accordingly, another pan-RXR (BMS649, 10^{-1} μM) also gave a similar ratio of metaplasia (data not shown).

When compared with *t*RA treatment, the degree of metaplasia was significantly higher after 10^{-3} μM pan-RAR treatment: about 45% of hair vibrissa follicles were changed into GG, and the AGG and duct types were never observed (**Table II**). Furthermore, the morphology of the GG was slightly different: glomerules were larger, with a wider lumen, and there were branched ducts like in salivary glands (**Fig 5*B***). Moreover, a further 45% of vibrissæ gave rise to a supplementary type of vibrissa metaplasia, characterized by a hyperdevelopment of the SG (**Fig 5*A*, *C*).** The most frequent transformation (about 30%) corresponded to a hyperdeveloped SG associated with a GG (GSG in **Table II).** A few hyperdeveloped SG were also found associated with an AGG, and some SG developed without any GG adjunction (not shown), thus resembling the SG present in the human ear and nose skin. Finally, the remaining 10% vibrissa follicles were all associated with abnormally developed SG. Although secretion granules were present in the lumen of all the glomerules (not shown), only a few ducts actually opened into the epidermis, most of them presenting a dead end. Increasing the concentration of the pan-RAR resulted in a nearly equal proportion of GG and GSG and a dramatic decrease of the SG ratio.

Agonists specific either for RARα or for RARγ, but not for RARβ, can initiate a glandular metaplasia of WT explants To establish if the activation of a single RAR is sufficient to induce a glandular metaplasia, WT explants were treated with three synthetic retinoids (Ro40–6055, BMS453, and CD437) displaying different transactivation abilities for the three RAR. At 8×10^{-3} μM, Ro40–6055 is believed to act as a RARα-specific agonist (Apfel *et al*, 1992, 1995). Nevertheless, it transformed about 28% of the vibrissæ into GG (**Fig 4*C***, **Table II**). Similarly, at 7.7×10^{-2} μM, a concentration at which it has been shown to specifically transactivate RARγ (Martin *et al*, 1992), CD437 gave rise to the same ratio of GG metaplasia (**Fig 4*D***, **Table II**). Strikingly, the treatment with both these molecules did not increase the ratio of vibrissa metaplasia (**Table II**); however, all the four explants responded when treated with both these molecules, whereas only five of six or four of five of the explants, respectively, treated with Ro40–6055 or CD437 gave rise to glandular metaplasia. On the other side, at 10^{-1} or 1 μM, BMS453 acts as a RARβ-specific agonist as well as a RARα and RARγ antagonist (Chen *et al*, 1995). As shown in **Table II**, none of the 13 explants treated with 10^{-1} μM BMS453, nor the two explants treated at the dose of 1 μM, exhibited any vibrissa metaplasia.

On a second occasion, we checked the effects of increasing the dose of Ro40–6055 or CD437. In both cases, increasing the dose enhanced the ratio of vibrissa metaplasia (**Fig 4*C*, *D*, Table II**), but should also lead to the lack of RAR specificity (Martin *et al*, 1992). Higher doses of Ro40–6055 gave rise to a hypertrophy of SG, so that, at 1 μM, the distribution of the GG, hyperdeveloped sebaceous glands associated with an AGG, and GSG was nearly identical to that previously obtained when using the CD367 pan-RAR at the same concentration. In contrast, explants treated with CD437 only displayed GG and AGG.

DISCUSSION

None of the RARα$^{-/-}$, β$^{-/-}$, and γ$^{-/-}$ null mutants presented any apparent defect in appendage morphogenesis. Consequently, none of the three RAR appeared to be absolutely required for a proper hair vibrissa follicle differentiation, at least in the surviving embryos. More precisely, it suggests that the two RAR detected in skin, i.e., the RARα and γ (Elder *et al*, 1991; Fisher *et al*, 1994; Viallet and Dhouailly,

Table I. RARγ knockout dramatically reduces the ratio of retinoic acid-induced glandular metaplasia[a]

Genotype	Explants[b]	Appendages[c]	% Vibrissæ[d]	% metaplasia[e]		
				AGG	D	GG
Wild-type	5 [5]	141 [28.2]	41.8 (±4.2)	17.7 (±3.2)	8.5 (±2.3)	31.9 (±3.9)
RARα$^{-/-}$	4 [4]	92 [23.0]	50.0 (±5.2)	13.0 (±3.5)	2.2 (±1.5)	34.8 (±5.0)
RARβ$^{-/-}$	4 [4]	98 [24.5]	50.0 (±5.1)	10.2 (±3.1)	9.2 (±2.9)	30.6 (±4.7)
RARγ$^{-/-}$	5 [3]	139 [27.8]	79.9 (±3.4)	9.4 (±2.5)	1.4 (±1.0)	9.4 (±2.5)

[a]For each embryo, one explant was treated with 16.5 μM all-*trans* retinoic acid, while the contralateral explant was treated with ethanol alone. No glandular metaplasia was observed in these controls.
[b]Number of treated explants. The number of explants having given rise to glandular metaplasia is indicated into brackets.
[c]Total number of appendages onto the treated explants. The mean number of appendages per treated explant are given in brackets.
[d]Per cent normal vibrissæ ±SD.
[e]Per cent transformed appendages ±SD. AGG, glomerular gland associated with a vibrissa; D, duct not connected to a glomerule; GG, glomerular gland.

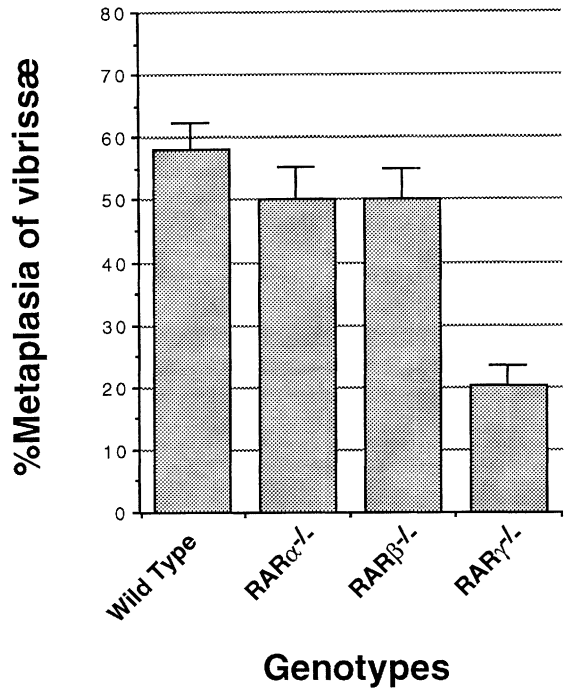

Figure 3. RARγ knockout dramatically decreases the response to *t*RA treatment. Bars represent the percentage of vibrissæ that were transformed into glands in explants of 13.5 d WT, RARα$^{-/-}$, RARβ$^{-/-}$, or RARγ$^{-/-}$ embryos treated for 2 d with 16.5 μM *t*RA and processed as in **Fig 2**. *Error bars*, mean ± SD.

1994b), can substitute for each other. This is in agreement with the presence of phenotypically normal hairs and whiskers in adult RARα$^{-/-}$, β$^{-/-}$, and γ$^{-/-}$ mice. This is further evidence of the functional redundancy previously observed between RARα and γ (Lufkin *et al*, 1993; Lohnes *et al*, 1993; Ghyselinck *et al*, 1997); however, as previously shown using RARα$^{-/-}$ or RARγ$^{-/-}$ knockout F9 cells, such a redundancy, which can be artificially generated by gene knockout, does not necessarily imply that these two receptors usually control the transcription of the same set of genes (Taneja *et al*, 1996).

RAR are the primary effectors of the upper-lip skin glandular metaplasia It is known that *t*RA can isomerize into 9-*cis* retinoic acid that binds RAR as well as RXR (Heyman *et al*, 1992; Levin *et al*, 1992; Allegretto *et al*, 1993); however, CD367, a pan-RAR with no effect onto RXR (Xiao *et al*, 1995), was able not only to induce the hair vibrissa metaplasia in all treated explants, but also to transform 100% of the vibrissæ into glands and to produce a SG hyperplasia, strongly suggesting that the RAR are the primary effectors of vibrissa metaplasia. That *t*RA treatment never gives rise to a SG hypertrophy may possibly arise from its rapid metabolism *in vivo*. Similarly, Feng *et al* (1997) established that *t*RA and the RAR-selective ligand CD367, but not the RXR-selective ligand SR11237, can induce the transcription of the CRABPII, CRBPI, and CRBPII in mouse epidermis *in vivo*. Under these conditions, why should the treatment with a pan-RXR (either Ro25–7386 or BMS649) also transform a few follicles of all treated explants into glands? It has been progressively established that RXR/RAR heterodimers are the functional units that preferentially transduce the retinoid signal to activate the expression of target genes (Chambon, 1996, and references therein), especially *in vivo* in the mouse (Kastner *et al*, 1997) and in cultures of human keratinocytes (Xiao *et al*, 1995). The concomitant administration of suboptimal concentrations of RAR- and RXR-specific ligands to cultured embryonal carcinoma P19 and F9 cells was shown to result in the synergistic activation of the expression of a number of endogenous retinoic acid-responsive genes (Roy *et al*, 1995; Taneja *et al*, 1996). It is thus conceivable that the pan-RXR treatment of the upper-lip skin may allow such a synergistic activation of RXR/RAR heterodimers together with the endogenous retinoids. Alternatively, the RXR were shown to function as promiscuous heterodimeric partners for several nuclear receptors, including the peroxysome proliferators-activated receptors, the liver X receptors, and the orphan receptor NGFI-B. Taking into account that the RXR are ligand responsive when heterodimerized with one of these receptors (Perlman and Janson, 1995; DiRenzo *et al*, 1997; Willy and Mangelsdorf, 1997), it is possible that the pan-RXR treatment could interfere with another receptor pathway. For example, Kang *et al (*1997) demonstrated that the 9-*cis* retinoic acid treatment of human skin can enhance the stimulation of the transcription of the 24-hydroxylase gene by the 1,25-dihydroxyvitamin D_3-liganded vitamin D receptor/RXR heterodimer. A further study of genes whose expression is perturbated in hair glandular metaplasia is thus obviously required to understand the molecular bases of this phenomenon; however, one major limitation in interpreting experiments with synthetic retinoids is that little is known about the variability in their uptake and/or their intracellular concentration in the different mouse embryonic or adult organs. Furthermore, activation of RAR by synthetic retinoids is not necessary functionally equivalent to activation by *t*RA.

RARγ, whose knockout significantly reduces the response to the *t*RA treatment, seems able to induce a glandular metaplasia without the need of RARα and β activation The treatment of RARα$^{-/-}$ explants with *t*RA indicated that RARγ is able to initiate a glandular metaplasia of vibrissæ, considering that no RARβ transcript can be detected in mouse upper-lip skin (Viallet and Dhouailly, 1994b). Only three RARγ$^{-/-}$ explants responded to the treatment, however, and moreover at a ratio and a degree (ratio of GG *versus* AGG) of vibrissa metaplasia significantly lower than the explants of the three other genotypes. This is in agreement with the fact that RARγ is believed to be much more abundant than RARα in mouse embryonic skin, as suggested by their respective transcript levels in WT mouse embryo (Viallet and Dhouailly, 1994b) and the dosage of the RAR in human (88% RARγ *versus* 12% RARα, Fisher *et al*, 1994). The lower response of the RARγ$^{-/-}$ upper-lip skin may also arise from the fact

Table II. RARα and γ are the primary effectors of retinoid-induced glandular metaplasia of vibrissæ

Retinoids (μM)		Explants[a]	Appendages[b]	% Vibrissæ[c]	% Metaplasia[d] AGG	D	GG	AGS	GSG	SG
CD367	10^{-3}	5 [5]	119 [23.8]	10.1 (±2.8)	–	–	43.7 (±4.5)	4.2 (±1.8)	29.4 (±4.2)	12.6 (±3.0)
	1	4 [4]	72 [18.0]	–	–	–	36.1 (±5.7)	4.2 (±2.4)	58.3 (±5.8)	1.4 (±1.4)[e]
	10	4 [4]	64 [16.0]	–	–	–	42.2 (±6.2)	7.8 (±3.4)	48.4 (±6.2)	1.6 (±1.6)[e]
Ro25-7386	10^{-3}	3 [3]	85 [28.3]	80.0 (±4.3)	8.2 (±3.0)	–	10.6 (±3.3)	–	–	1.2 (±1.2)[e]
	1	3 [3]	85 [28.3]	36.5 (±5.2)	21.2 (±4.4)	–	29.4 (±4.9)	1.2 (±1.2)[e]	11.8 (±3.5)	–
	10	4 [4]	91 [22.8]	68.1 (±4.9)	18.7 (±4.1)	–	12.1 (±3.4)	–	1.1 (±1.1)[e]	–
Ro40-6055	10^{-3}	3 [0]	48 [16.0]	100	–	–	–	–	–	–
	8×10^{-3}	6 [5]	115 [19.2]	72.2 (±4.2)	8.7 (±2.6)	1.7 (±1.2)	17.4 (±3.5)	–	–	–
	10^{-2}	7 [7]	142 [20.3]	69.7 (±3.9)	19.7 (±3.3)	–	10.6 (±2.6)	–	–	–
	10^{-1}	7 [7]	118 [16.9]	20.3 (±3.7)	58.5 (±4.5)	–	13.6 (±3.2)	1.7 (±1.2)	5.1 (±2.0)	0.8 (±0.8)[e]
	1	5 [5]	102 [20.4]	1.0 (±1.0)	10.8 (±3.1)	–	30.4 (±4.6)	2.9 (±1.7)	54.9 (±4.9)	–
BMS453	10^{-3}	4 [0]	93 [23.3]	100	–	–	–	–	–	–
	10^{-2}	3 [0]	69 [23.0]	100	–	–	–	–	–	–
	10^{-1}	13 [0]	271 [20.8]	100	–	–	–	–	–	–
	1	2 [0]	41 [20.5]	100	–	–	–	–	–	–
CD437	10^{-3}	6 [0]	140 [23.3]	100	–	–	–	–	–	–
	10^{-2}	6 [0]	119 [19.8]	100	–	–	–	–	–	–
	7.7×10^{-2}	5 [4]	110 [22.0]	71.8 (±4.3)	11.8 (±3.1)	1.8 (±1.3)	14.5 (±3.4)	–	–	–
	10^{-1}	5 [5]	127 [25.4]	89.8 (±2.7)	7.1 (±2.3)	–	3.1 (±1.5)	–	–	–
	1	5 [5]	127 [25.4]	25.2 (±3.9)	70.1 (±4.1)	–	4.7 (±1.9)	–	–	–
Ro40-6055 + CD437[f]		4 [4]	85 [21.3]	76.5 (±4.6)	9.4 (±3.2)	–	14.1 (±3.8)	–	–	–

[a]Number of treated explants. The number of explants having given rise to glandular metaplasia is indicated into brackets.
[b]Total number of appendages onto the treated explants. The mean number of appendages per treated explant are given into brackets. For each embtyo, one explant was treated with the indicated retinoid, while the contralateral explant was treated with DMSO alone. No glandular metaplasia was observed in these controls.
[c]Per cent normal vibrissæ ±SD.
[d]Per cent transformed appendages ±SD. AGG, glomerular gland associated with a vibrissa; D, duct not connected to a glomerule; GG, glomerular gland; SG, hupertrophied sebaceous gland; GSG, glomerular gland associated with a SG; AGS, AGG associated with a SG.
[e]One unique appendage displayed this type of metaplasia.
[f]Explants were simultaneously treated with 8×10^{-3} μM Ro40-6055 and 7.7×10^{-2} μM CD437.

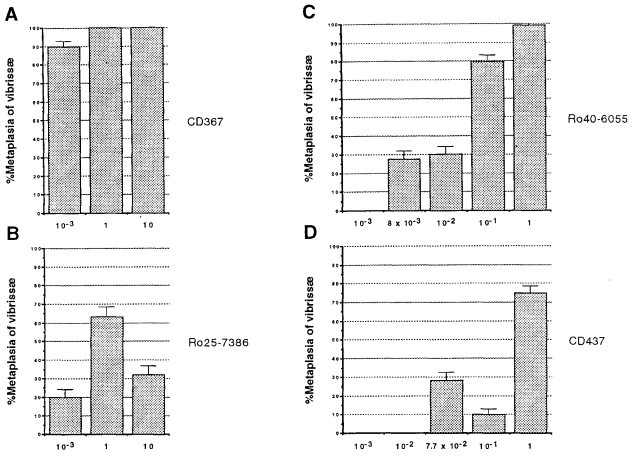

Figure 4. The activation of more than one RAR is required to reach the highest ratios of vibrissa metaplasia. WT explants were treated with (*A*) the CD367 RAR pan-agonist; (*B*) the Ro25–7386 RXR pan-agonist; (*C*) Ro40–6055, believed to be RARα-specific at 8×10^{-3} μM; (*D*) CD437, believed to be RARγ-specific at 7.7×10^{-2} μM. Bars represent the percentages of vibrissæ transformed into glands in WT 13.5 d upper-lip skin explants treated for 2 d with the indicated retinoid and processed as in **Fig 2.** Concentrations are given in μM. *Error bars*, mean ± SD.

that, although RARα and γ transcripts are both present in the dermis and epidermis of 13.5 d WT embryos, RARα transcripts are no longer detectable in these tissues 2 d later (Viallet and Dhouailly, 1994b), suggesting that RARα is not present in embryonic skin at this stage of development. Thus, at this moment, the RARγ knockout may lead to the lack of RAR in embryonic skin, except a low level of *t*RA-induced RARβ. Furthermore, taking into account that the follicles of one given explant are not at the same stage of development and can only respond during a narrow temporal window (stages 1–3), one can imagine that the RARγ knockout may either reduce this window or impair the response of the more advanced vibrissæ.

At 7.7×10^{-2} μM, the CD437 retinoid is believed to be specific for RARγ (Martin *et al*, 1992); however, it truely induced a glandular metaplasia at this dose, thus suggesting that the activation of the two other RAR, especially RARα, also present in skin, is not an absolute requirement. Nevertheless, we cannot rule out that some activation of RARα may also have occurred through the endogenous retinoids. In any case, even at 1 μM, the CD437 RAR selective-retinoid transformed no more than 75% of vibrissæ into glands, and the ratio of GG *versus* AGG was below 1. Furthermore, no vibrissa displayed SG hypertrophy after this treatment. These results, which are different from those obtained using the CD367 pan-RAR, suggest that the highest ratios and degrees of metaplasia, as well as the SG hypertrophies, require the activation of at least two RAR.

The RARα is able to initiate a glandular metaplasia of vibrissæ, at least when the RARγ is absent or not activated Taking into account the lack of RARβ in mouse upper-lip skin, the *t*RA treatment of $RAR\gamma^{-/-}$ explants unambiguously demonstrated that RARα is able to initiate a glandular metaplasia of vibrissæ, at least in the absence of RARγ. This result is confirmed by the observation that the treatment of WT explants with 8×10^{-3} μM Ro40–6055 (a concentration at which this retinoid is believed to be specific for the RARα, Apfel *et al*, 1992, 1995) also induced this metaplasia. Increasing the dose of Ro40–6055 from 8×10^{-3} to 10^{-1} μM concurrently increased the ratio of vibrissa metaplasia from about 30% to 80%, but the ratio of GG did not grow accordingly, so that the ratio of GG *versus* AGG decreased from 2 to 0.2. Interestingly, 10^{-1} μM Ro40–6055 also induced a SG hypertrophy. At the highest used dose (1 μM), the ratios of both the GG and the GSG were nearly identical with those obtained when treating the WT explants with the same concentration of the CD367 pan-RAR. We must obviously keep in prudence with the interpretation of receptor-selective ligand effects, considering that the variability in the uptake and/or the intracellular metabolism of the different retinoids in mouse embryonic or adult skin is unknown. As

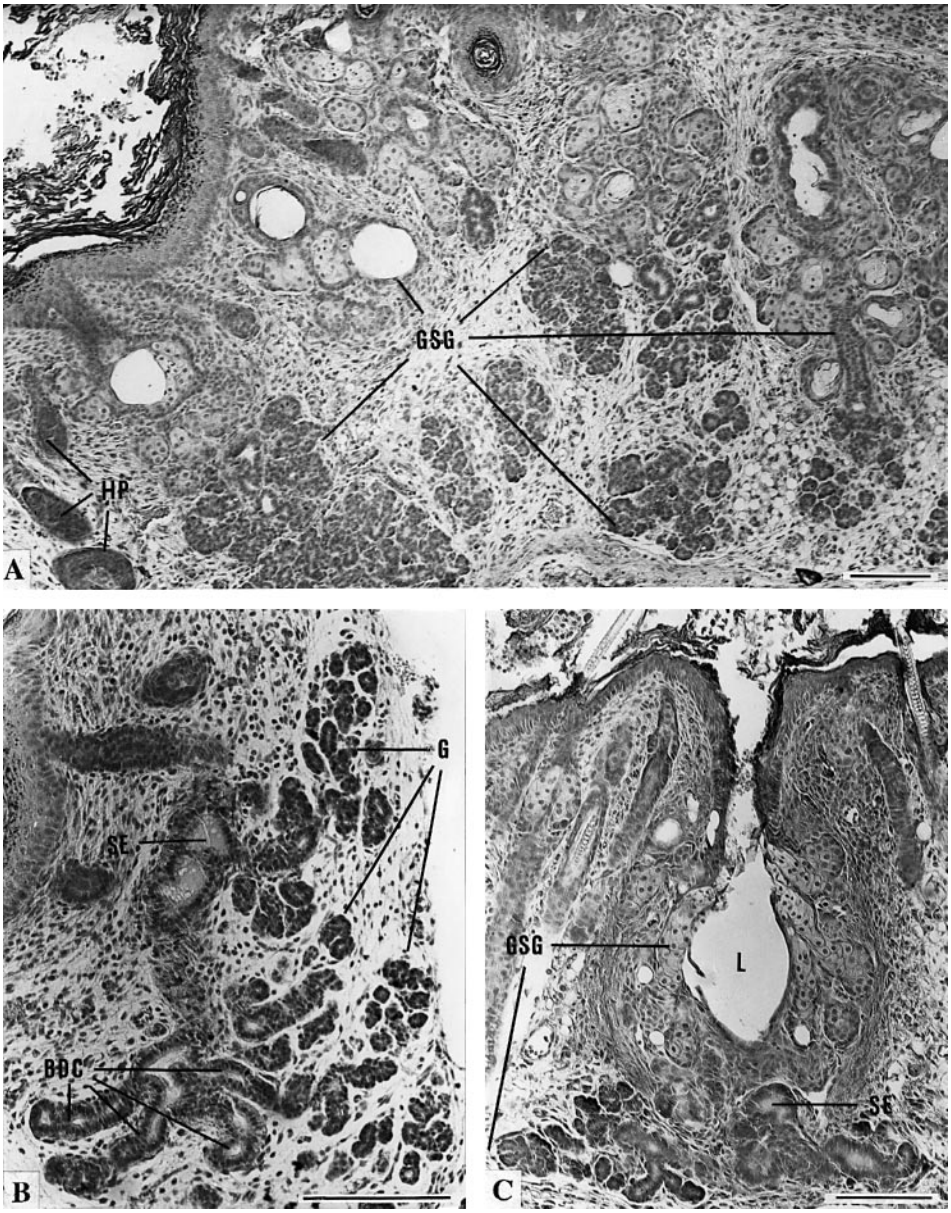

Figure 5. Treatment with a RAR pan-agonist induces not only a hair vibrissa metaplasia, but also a sebaceous gland hypertrophy. Explants of 13.5 d upper-lip skin were treated for 2 d with the CD367 RAR pan-agonist (10^{-3} μM) and processed as in **Fig 2**. (*A*) Overview showing several glomerular glands associated with GSG and a complete disappearance of hair vibrissa follicles. Only some hair pelage follicles (HP) are formed (compare with the control, **Fig 1*B***). (*B*) Detail of a glomerular gland with a branched duct (BDC) and secretories elements (SE). (*C*) Detail of a GSG. G, Glomerule; L, lumen. (*A–C*) Hematoxylin/Biebrich scarlet staining. *Scale bar*, 100 μm.

the Kds of Ro40–6055 for RARα, β, and γ are, respectively, 8, 131, and 450 nM (Apfel *et al*, 1992, 1995), however, it is probable that at least a part of the effects obtained at the highest concentrations of Ro40–6055 arose through the activation of one of the two other RAR, or both. In other words, Ro40–6055 should progressively behave as a pan-RAR in proportion as the dose is increased.

RARβ-specific agonist cannot initiate a glandular metaplasia, but the RARβ may act synergistically with the two other RAR to increase the degree of metaplasia None of the explants treated with the BMS453 RARβ-specific agonist gave rise to a glandular metaplasia. This result is in agreement with the lack of RARβ transcripts in embryonic mouse upper-lip skin (Viallet *et al*, 1991; Viallet and Dhouailly, 1994b). This suggests that the RARβ is not required for the induction of glandular metaplasia, as already shown when *t*RA-treated heterotopic recombinants of upper-lip epidermis and dorsal dermis resulted in glands formation without induction of RARβ transcription (Viallet and Dhouailly, 1994b); however, it should be reminded that *t*RA induces the transcription of the RARβ gene in upper-lip skin (Viallet *et al*, 1991). The fact that the highest degrees and ratios of metaplasia as well as a SG hyperdevelopment were obtained by treatment either with CD367 or with Ro40–6055 (only at the highest doses for the latter), but not with a combination of Ro40–6055 and CD437 at the concentration where these two molecules are believed to be, respectively, specific for RARα and RARγ, strongly suggests that RARβ activation may also be required. Unfortunately, it was not possible to test directly with the WT explants whether RARβ should act synergistically with either RARα or RARγ, or both these receptors, because the BMS453 RARβ-specific agonist also displays a RARα- and RARγ-antagonistic activity (Chen *et al*, 1995). This question remains to be addressed by using other retinoids, or, more rigorously, by treating explants from the different null mutants with a pan-RAR. It also remains to identify the genes whose expression is perturbated following retinoid exposure of mouse upper-lip skin.

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