Supplementary data: Materials and Methods

Mouse lines and genotyping

All mice were on a mixed 129/Sv-C57BL/6 genetic background. Mice carrying RARα null, RARγ null, and loxP-site-containing (floxed) RXRα alleles, as well as CMV-Cre-ERT and K5-Cre-ERT mouse lines have been described (Lufkin et al., 1993; Lohnes et al., 1993; Li et al., 2000; Brocard et al., 1997; Indra et al., 1999). Floxed RARα and RARγ alleles are described elsewhere (Chapellier et al., 2002a and 2002b). Tail epidermis was separated from dermis after treatment with dispase (4 mg/ml in PBS; Gibco-BRL) for 1-2 hours at room temperature. Genomic DNA was isolated by standard methods. Analysis of RXRα L2 or L−, RARα L2 or L− and RARγ L3 or L− alleles, as well as RARα and RARγ genotyping were as described (Lufkin et al., 1993; Lohnes et al., 1993; Li et al., 2000; Chapellier et al., 2002a and 2002b).

Tamoxifen and retinoid treatment

Tamoxifen (TAM; Sigma) was injected intraperitoneally (1 mg in 100 µl sunflower oil) according to experimental protocols depicted in figures. TAM treatment on its own had no effect on epidermis proliferation of wild-type (WT) and other control mice (data not shown). Synthetic agonist retinoids selective for RARα (BMS753) and RARγ (BMS961), as well as the panRXR agonist (BMS649) were gifts from Bristol-Myers Squibb (Wallingford, CT). Their selectivity was assessed by transactivation and direct binding assays (Chen et al., 1995b; Gehin et al., 1999; our unpublished
observations). BMS649 is identical to SR11237 (Gendimenico et al., 1994). All trans RA (RA, Sigma) and BMS compounds were diluted in acetone immediately before use. Prior to topical retinoid treatment, hairs were removed by shaving isoflurane-anaesthetized mice using electric clippers. A single daily dose of retinoids (40 nmoles) was applied topically (400 µl) on dorsal skin over an area of approximately 6 cm$^2$ for a period of 4 days. Each experiment (2 to 3 weight-matched mice of each genotype simultaneously treated) was repeated at least 3 times. Females (8-12 week-old) were used, as males were less responsive to RA-induced epidermal hyperproliferation (our unpublished observations). Acetone vehicle treatment on its own did not affect epidermal proliferation (data not shown).

**Histology and proliferation analysis**

Skin samples were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2), embedded in Epon 812, sectionned at 2 µm and stained with toluidine blue. To detect proliferating cells, mice were injected intraperitoneally with 5-bromo 2'-desoxyuridine (BrdU; 50 mg/kg body weight) and skin biopsies were performed 2 hours later, fixed in Bouin's fluid and embedded in paraffin. Sections (7 µm thick) were incubated with an anti-BrdU monoclonal antibody (Boehringer Mannheim), and revealed using Vectastain ABC and peroxidase substrate DAB kits (Vector). Sections were counterstained with Harris hematoxylin. Alternatively, immunolocalization of the proliferation marker Ki67 (Schlüter et al., 1993) was determined. Frozen sections (10 µm thick) from unfixed skin samples were post-fixed in 2% paraformaldehyde (PFA) in sodium phosphate buffer (PBS), for 10 minutes at room temperature (RT).
The antigen was unmasked by high temperature treatment in 10 mM sodium citrate buffer pH 6.0 and the slides were incubated for 2 hours at RT with an antibody against Ki67 diluted 1/500 in PBS (according to Novocastra’s protocol). CY3-conjugated anti-rabbit IgG (Jackson Immunoresearch), diluted 1/400 in PBS was used as secondary antibody. Counterstaining was performed with 0.01% DAPI (4’, 6-diamidino-2-phenylindole dihydrochloride, Boehringer Mannheim) in Vectashield mounting medium (Vector). For each mouse, the number of either BrdU-labelled or Ki67-positive and total basal keratinocytes were counted on 5 high-power fields in interfollicular areas. Data from the 5 areas were pooled, and the percentage of BrdU-labelled or Ki67-positive basal cells over total basal cells (>200) was calculated. Means ± SD were estimated by analyzing skin samples from at least 3 animals of each genotypes.

**Immunohistochemistry**

Distribution patterns of keratins 5, 6, 10 and 13 were determined as described (Li et al., 2000). For RXRα immunodetection, skin samples were fixed for 6 hours at 4°C with periodate-lysine-paraformaldehyde (MacLean and Nakane, 1974) and dehydrated in graded sucrose. Unspecific binding of the antibody was prevented by incubating frozen sections with 10% heat-inactivated goat serum (NGS) for 30 minutes at RT. Sections were then incubated overnight at 4°C with the RXRα monoclonal antibody 4RX3A2 (Rochette-Egly et al., 1994) diluted 1/4000 in PBS. Biotinylated anti-mouse IgG (Vector), diluted 1/400 in PBS was used as secondary antibody. RARγ immunolocalization was determined as described (Ghyselinck et al.,
1997). Samples from RXRα null fetuses (Kastner et al., 1994) and RARγ null mutants (Lohnes et al., 1993) were used as immunostaining negative controls.

**RNA analyses**

Total RNA were prepared using Trizol reagent (Gibco-BRL). Mouse CRABPII and RARβ2 probes have been described elsewhere (Ruberte et al., 1992; Lohnes et al., 1993). Mouse β-actin cDNA probe was from Clontech. To prepare HB-EGF cDNA probe, mouse epidermal RNA was reverse-transcribed and amplified by PCR using primers 5’-AAGTGATCGCTGCCTCCCCGTCTCC-3’ (sense, position 187; Genbank L07264) and 5’-CAAGTCATAACCTCCTCTCCTCTGTGCG-3’ (antisense, position 783). The resulting 621 bp-long cDNA fragment was purified and verified by sequencing. Northern blotting (25 µg total RNA per lane) and hybridizations were carried out using standard protocols. For quantitation of mRNA, the blots were analyzed with a Phosphor-Imager (Fuji). Signals were quantified and normalized against the corresponding β-actin signals. For RNAse protection assay, 20 µg total RNA was used per reaction. The RARβ2 riboprobe protected fragments of 336 bp for the RARβ2 isoform and of 260 bp for all other RARβ isoforms. The histone H4 antisense riboprobe used as an internal control generated a 130 bp fragment (Lohnes et al., 1993). Total RNA from RA-treated F9 teratocarcinoma cells was used as positive control.

**References**


