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A *PMLRARA* Transgene Results in a Retinoid-deficient Phenotype Associated with Enhanced Susceptibility to Skin Tumorigenesis[1]

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ABSTRACT

The construction of transgenic FVB/N mice targeting the *PMLRARA* fusion gene under the control of a human *MRP8* promoter recapitulated the phenotype of acute promyelocytic leukemia but had the unexpected result of multiple squamous papillomas of the skin (Brown *et al., Proc. Natl. Acad. Sci. USA*, 94:2551–2556, 1997). In addition, transgenic *MRP8-PMLRARA* mice exhibited a skin phenotype characteristic of vitamin A deficiency. The severity of the skin phenotype and spontaneous papilloma development correlated with the level of transgene expression. Papilloma formation was preceded by follicular hyperplasia and the expression of epidermal differentiation markers in the follicular epithelium. Mutations in the Ha or Ki alleles of *ras* were not detected in papillomas that developed on transgenic skin, and papilloma formation was accentuated on the C57/Bl6 background, unlike the usual resistance of this strain to skin tumor induction. Analysis of liver extracts from transgenic mice indicated a deficiency in the production of retinoic acid. Furthermore, affected transgenic epidermis had reduced levels of retinoic acid receptorα (RARα) and retinoic X receptor (RXRα), and supplementation with exogenous retinoic acid prevented the skin phenotype. When transgenic keratinocytes were grafted to nude mice, the resulting integument was normal, and conversely, when transgenic bone marrow was grafted to normal mice, a skin phenotype did not develop. Together these results suggest that local interruption of PML and RARα signaling in the skin, together with a systemic retinoid deficiency, initiates a tumor induction pathway that is independent of *ras* activation.

INTRODUCTION

Fusion of most of the *RARA* and *PML* genes via a reciprocal chromosomal translocation, thus creating the *PMLRARA* fusion gene, occurs in the majority of APL[3] cells (1–3). Several laboratories have developed *PMLRARA* transgenic mice as a model for APL or to analyze the biological properties of the fusion gene (4–9). The PML-RARα protein exerts dominant-negative effects on both PML, a nuclear body protein that can limit cell growth and survival, and RARα signaling (2). Consequently, interrupted signaling prevents full maturation of promyelocytes resulting in the accumulation of immature leukemic cells. PML-RARα can homodimerize or form heterodimers with both PML and RXRs (reviewed in Refs. 1, 2). PML-RARα oligomers or PML-RARα RXR dimers cause transcriptional repression of RAR-RXR signaling by recruiting histone deacetylase together with the transcriptional corepressors SMRT and N-CoR to RAREs of target genes (10–13). Because PML-RARα is typically expressed more highly than other retinoid receptors, whereas retaining an affinity for RXR that is similar to RARs, PML-RARα expression also results in sequestration of RXRs from RARs (2, 14). PML-RARα binds ATRA with similar affinity as RARα, although RARE-mediated transcription occurs only on pharmacological doses of ATRA (2, 15). Pharmacological doses of ATRA cause degradation of the fusion protein as well as up-regulation of RARα activity resulting in restoration of the normal function of both RARα and PML, and full maturation of the leukemic cells (reviewed in Ref. 2). In APL patients, disease remission in response to ATRA therapy is believed to be a consequence of ATRA-induced differentiation of leukemic cells (reviewed in Ref. 1). Thus, PML-RARα signaling appears to modulate cell differentiation and tumorigenesis, at least in part, by dysregulating retinoid signaling pathways.

PML-RARα also represses PML-modulated transcriptional regulation by forming heterodimers with PML and altering its subcellular localization. PML acts as a tumor suppressor by regulating cell growth and apoptosis. PML also interacts with viral oncogenes (2). These functions for PML are apparent in APL cells as well as in the skin. PML nullizygous mice exhibit enhanced susceptibility to chemical carcinogen-induced skin carcinogenesis (16). In APL cells, gene dosage of PML correlates with the latency of APL onset in *PMLRARA* transgenic mice (17).

A murine model for APL, developed in one of our laboratories, used the *MRP8* promoter to target the human fusion gene to bone marrow (9). MRP8 was originally described as a member of the S100 family of proteins that is expressed during differentiation of myelomonocytic cells (18) but was subsequently found expressed in differentiating epithelial cells including skin keratinocytes (19). During the course of the analysis of the *MRP8-PMLRARA* mouse, a marked sensitivity to spontaneous skin tumor formation, often limiting the life span of these animals, was noted. Retinoids are well known to have a major influence on skin differentiation and tumor formation, and retinoid receptors are modified in both murine and human cutaneous neoplasms (20–24). Furthermore, genetic ablation of RXRα results in a marked alteration in skin development (24). We now report an additional characterization of the skin phenotype of the *MRP8-PMLRARA* mouse, and correlate that to transgene expression and retinoid response. The results implicate both PML function and retinoid metabolism as contributing to the sensitivity to skin tumor induction, thus illuminating a novel pathway leading to cutaneous neoplasia.

MATERIALS AND METHODS

Animals. Transgenic mice of the FVB/N strain were created using a human *MRP8* promoter and a human *PMLRARA* cDNA with a chromosome 15 breakpoint in breakpoint cluster region 1 (9, 25). Tail DNA was prepared as described elsewhere (9) for genotyping using PCR. ATRA was administered to mice via s.c. implantation of a 21-day-release pellet containing 5 mg ATRA or placebo (Innovative Research of America, Sarasota, FL). For wounding experiments, a wound clip was attached to the back skin of 6–8-week-old mice. After 12 days, the wound clip was removed, and tumor development was monitored weekly for 6 weeks.

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[3] The abbreviations used are: APL, acute promyelocytic leukemia; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element; *MRP*, migration inhibitory factor-related protein; ATRA, retinoic acid.

Cell Culture and Grafting. Eagle's MEM and penicillin-streptomycin were obtained from Life Technologies, Inc. (Gaithersburg, MD), and FCS was from Gemini Bio-Products (Calabasas, CA). Primary keratinocytes were obtained from newborn transgenic *PMLRARA* and littermate controls. Keratinocytes were prepared as described previously (26) and cultured in calcium- and magnesium-free Eagle's MEM with 8% chelexed (Bio-Rad Laboratories, Hercules, CA) serum, 20 units/ml penicillin, 20 μg/ml streptomycin in Eagle's MEM, and 0.05 mM calcium chloride. Cells were initially plated in medium adjusted to 0.25 mM calcium and changed to 0.05 mM calcium-containing medium ~18 h later. Viral infection with a v-*ras*^{Ha} replication-defective retrovirus was performed using diluted supernatant from ψ-2 producer cells in the presence of 4 μg/ml Polybrene (27). Grafting of *MRP8-PMLRARA* and nontransgenic keratinocytes with or without v-*ras*^{Ha}-expression together with primary BALB/c dermal fibroblasts onto athymic nude mice was performed as described previously (26). Tumor volume was measured weekly using digital calipers. Sequence analysis of tumor DNA for mutations in the *ras* ^{Ha} or *ras*^{Ki} alleles was performed as described previously (28).

Immunohistochemistry and Immunofluorescence. Skin and skin tumors were removed from 4, 8, 12, and 16-week-old FVB/N and *PMLRARA* transgenic mice after euthanasia, fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned. For immunofluorescence experiments, sections were incubated with polyclonal rabbit antibodies recognizing mouse keratin 1 (K1), loricrin, filaggrin, keratin 6 (K6), and keratin 10 (K10; Covance, Berkeley, CA). After incubation with the primary antibodies, sections were incubated with an antirabbit secondary antibody conjugated to fluorescein (Vector Labs.) and then counterstained using 4′,6-diamidino-2-phenylindole mounting medium (Vector Labs.). To detect transgene expression, sections were incubated with an antihuman PML antibody (PG-M3 from Santa Cruz Biotechnology), biotinylated anti-F(ab)$_2$ fragment antimouse secondary antibody (Jackson Immunoresearch Labs), horseradish peroxidase-conjugated streptavidin (Jackson Immunoresearch Labs), diaminobenzidine substrate, and counterstained with hematoxylin (Sigma).

Immunoblotting. Dorsal skin from transgenic and control mice was depilated with Nair, excised, and quick frozen. Minced frozen skin was extracted in 4°C buffer containing 500 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, 0.32 mM sucrose, 5 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride by polytron homogenization for 1 min. Fat and insoluble keratinous material was removed by filtration through 40 μm of mesh. The 1500-g pellet was re-extracted in buffer, and the 5000-g pellet was dissolved in Laemmli buffer and centrifuged at 75 K rpm for 15 min. The protein concentration of the supernatants was assayed, and equalized proteins were separated on 4–12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using polyclonal antibodies against RARα (C20; Santa Cruz Biotechnology; 1:1000), RXRα (D20; Santa Cruz Biotechnology; 1:1000), and RARα (RPα F-115; 1:5000) that recognizes both RARα and PML-RARα (29, 30), and was kindly provided by Dr. Pierre Chambon. Monoclonal antiactin antibody (Roche; 1:1000) was used as a loading control. Secondary antibodies were goat antirabbit and goat antimouse IgG-horseradish peroxidase conjugates (Bio-Rad), and visualization was performed with the Supersignal enhanced chemiluminescence kit from Pierce. For densitometry, images were quantified on Imagemaster (Amersham).

Analysis of Retinyl Palmitate Levels in the Liver of Control and Transgenic Mice. Livers were removed from age-matched FVB/N control and *PMLRARA* transgenic mice after euthanasia, and flash frozen in liquid nitrogen. Extraction and quantification of retinyl palmitate levels by high performance liquid chromatography was performed as described (31).

Bioassay for Synthesis of RA. Endogenous retinal dehydrogenase activity and retinal levels were quantified in age-matched *PMLRARA* transgenic and nontransgenic FVB/N livers using an assay modified from procedures described previously (32, 33). Livers were homogenized in 10 mM potassium phosphate buffer (pH 7.2; Mallinckrodt) with a mixture of protease inhibitors (complete Mini, EDTA; Roche) and 1 mM pepstatin (Roche), followed by sonication and two freeze/thaw cycles and centrifugation. Supernatant protein was quantified using a Micro BCA protein assay reagent kit (Pierce). A 3-h preincubation mixture with or without homogenate protein consisted of 2 mM DTT in L15-CO2 medium (Specialty Media) with 2.4 mM NAD and 20 nM retinal (or solvent control) to allow for the oxidation of retinal to RA by the endogenous retinaldehyde dehydrogenase. The incubated mixture was added to SIL-15 RA reporter cells transfected with a β-galactosidase gene under control of the RARE for RARβ. These cells were prepared by Dr. Michael Wagner and obtained from Dr. Peter McCaffery (Peter Bent Brigham Hospital, Boston, MA; Ref. 34). The reporter cells were cultured overnight in L15-CO2 medium with FVM and 1:1:2 supplements, 10% fetal bovine serum, 1× antibiotic/antimycotics, and 0.8 mg/ml Geneticin. Reporter cells were also treated with RA standards ranging in concentration from 0.125 nM to 1 nM with 8 replicates each to establish a standard curve. The plates were washed with PBS, fixed with glutaraldehyde, washed again with PBS, and incubated at 37°C for 3–5 h in development buffer consisting of 0.2% 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Promega), 1 mM $MgCL_2$ (Sigma), 3.3 mM $K_3Fe(CN)_6$ (potassium ferricyanate; Sigma), and 3.3 mM $K_4Fe(CN)_6$ (potassium ferrocyanate; Sigma) in PBS, and absorbance was measured at 630 nm.

Transplantation of Bone Marrow Cells. Total bone marrow isolated from the tibiae and femurs of a single donor was divided for i.v. injection into six recipient mice. Five to 12-week-old FVB/N mice were prepared for transplantation by cesium irradiation totaling 10.5 Gy, divided into two doses 3–6 h apart.

RESULTS

***MRP8-PMLRARA* Transgenic Mice Display a Hair Phenotype, Epidermal Thickening, and Spontaneous Papillomas at Sites of Wounding.** A skin phenotype that varied in severity among founder lines was observed in 10 of 11 founder transgenics and their offspring. The abnormalities varied from slight thickening of the skin at the site of the ear tag (Fig. 1*B*) to regions of thickened, hairless, corrugated skin, to many distinct papillomas covering a large portion of the body of the animal (Fig. 1, *A*, *C*, *E*, and *F*). Regions of thickly furrowed hyperkeratotic skin resolved frequently into thin hairless patches. *PMLRARA* transgenic mice developed grossly visible papillomas as early as 3 weeks of age (data not shown). Although rare, progression to carcinoma was detected. In the line with the highest transgene expression, line 556, transgenic pups had a sparser haircoat (Fig. 1*A*), which clearly distinguished them from nontransgenic littermates. Transgenic mice also exhibited scaly skin, particularly around the snout, and periorbital reddening (Fig. 1*C*), features characteristic of retinoid deficiency (35, 36).

Papilloma development was often associated with sites of bite wounds (Fig. 1*E*) or inflammation, such as the site of the ear tag (Fig. 1*C*). To verify that wounding could trigger tumor formation, wound clips were attached to the back skin of 6-week-old FVB/N control and *PMLRARA* transgenic mice as shown in Fig. 2*A*. The skin at the clip site was edematous in the FVB/N mice (Fig. 2*B*), whereas a tumor was already visible in the transgenic mice (Fig. 2*D*) when clips were removed 12 days later. Six weeks after placement of the clip, the site had returned to normal in the FVB/N mice (Fig. 2*C*), but the transgenic mice had sizeable tumors (Fig. 2*E*).

Histologically, regions of hyperplasia characterized the hair follicles and epidermis of the transgenic mice before the appearance of papillomas (Fig. 3). The interfollicular epidermis displayed patchy hyperplasia with occasional apoptotic or dysplastic cells (Fig. 3; data not shown). In 11-day-old pups, fewer hair follicles appeared to breach the surface of the epidermis, perhaps explaining the sparser hair coat, and the interfollicular epidermis was frequently thicker, although the overall pattern of differentiation, from basal cells to cornified cells, was intact (data not shown). In older mice, follicular hyperplasia was apparent, particularly in the upper, permanent portion of the hair follicle (Fig. 3), and excessive keratinization in the follicle ducts preceded papilloma formation (Fig. 3; data not shown).

Transgene Expression and Localization Correlates with the Skin Phenotype. Both the penetrance and expressivity of this phenotype were in accord with the levels of transgene expression seen in the bone marrow. All of the mice from the high-expressing line 556 had visible skin lesions by 6 weeks of age, and their lesions were

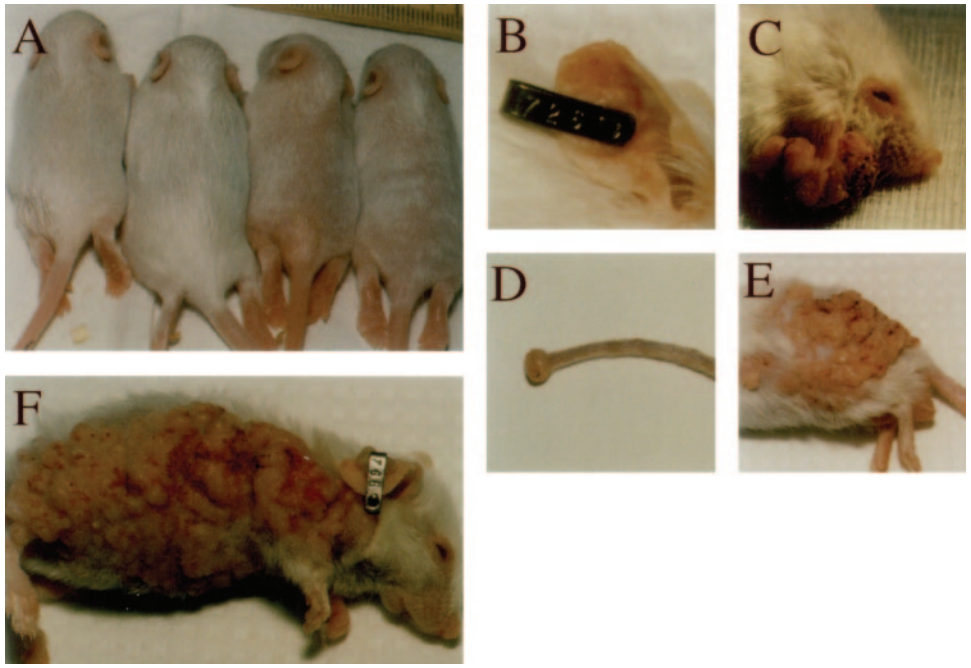

Fig. 1. MRP8-PMLRARA transgenic mice exhibited cutaneous vitamin A deficiency and spontaneous or wound-induced skin papilloma development. Eleven-day-old line 556 transgenic pups (pair on *right*) exhibit thinner, more irregular hair coat compared with nontransgenic littermates (pair on *left*; *A*). In older transgenic mice (*B–F*), periorbital erythema, scaling and edema (*C* and *F*), thickening of the skin (*B*), and skin tumors (*C–F*) were common at sites of the ear tag (*B*), tail biopsy (*D*), and bite wounds (*E*).

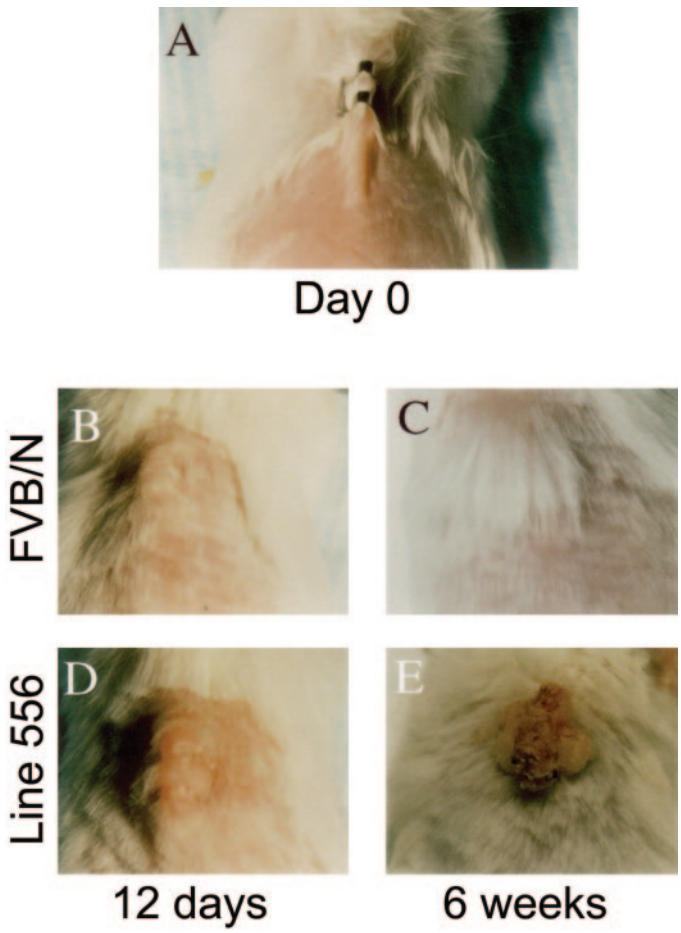

Fig. 2. Wounding induces papillomas on PMLRARα transgenic mice. A wound clip was attached to the back skin of 6–8-week-old mice (*A*). After 12 days, the wound clip was removed. The skin at the clip site was edematous in the FVB/N mice (*B*), whereas a tumor was already visible in the transgenic mice (*D*). Six weeks after placement of the clip, the site had returned to normal in the FVB/N mice (*C*), but the transgenic mice had sizeable tumors (*E*).

generally more severe than those seen in the low-expressing lines (Figs. 1 and 4). Mice bred to have increased transgene dosage generally had more severe phenotypes, as shown for lines 553 and 565 in Fig. 4.

Immunohistochemical analysis using an antihuman PML antibody that does not cross-react with mouse PML revealed *PMLRARA* transgene expression in the cutaneous epithelium, particularly in the follicular epithelium (Fig. 5). In hyperplastic epidermis, the transgene was expressed in the stratum granulosum as well. Expression was increased in papillomas with the strongest levels in the stratum granulosum (Fig. 5). Within the limits of immunohistochemical detection, transgene expression was consistently nuclear. No *PMLRARA* expression was detected in nontransgenic mouse skin using the antihuman antibody (data not shown). Taken together, these results strongly support the hypothesis that the skin lesions were associated with transgene expression.

Hair Follicles in *PMLRARA* Transgenic Mice Exhibit Epidermis-type Differentiation. Sebaceous and keratinizing differentiation were observed histologically in hyperplastic hair follicles from transgenic mice, in apparent association with the expression of the *PMLRARA* transgene in the hair follicles. Differentiation of the cutaneous epithelium was examined by immunohistochemistry using several well-characterized markers of epidermal differentiation. Expression of early markers of epidermal differentiation, keratins 1 and 10, was restricted to suprabasal stratum spinosum of the interfollicular epidermis in control FVB/N skin as shown in Fig. 6. In *PMLRARA* mice, in contrast, expression of K1 and K10 were localized not only to stratifying stratum spinosum, but aberrantly extended into the follicular epithelium (Fig. 6). K10 expression was detected in the upper, permanent portion whereas the entirety of many hair follicles was K1 positive (Fig. 6). Filaggrin, expressed in the stratum granulosum of the interfollicular epidermis as seen in skin from control FVB/N mice, was increased in the hyperplastic transgenic skin and abundant in transgenic follicles (Fig. 6). The pattern of differentiation markers in transgenic skin suggested that *PMLRARA* expression caused an epidermalization of hair follicles in association with the development of follicular papillomas.

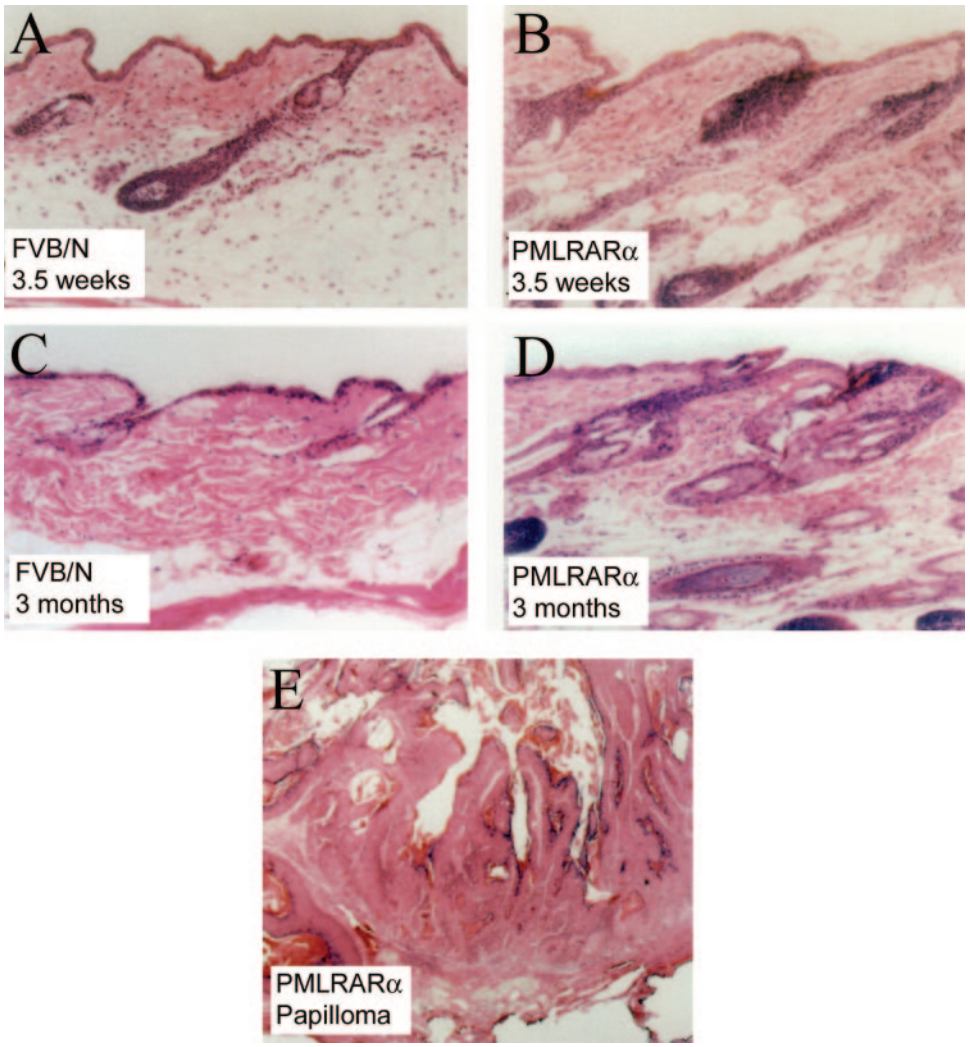

Fig. 3. PMLRARα transgenic skin exhibits follicular hyperplasia before papilloma development. Histology from nontransgenic (*A* and *C*) and PMLRARα transgenic mice (*B, D,* and *E*) of a representative spontaneous papilloma (*E*), skin from 3.5-week-old mice (*A* and *B*) and skin from 3-month-old mice (*C* and *D*) after H&E staining revealed that follicular hyperplasia predated the appearance of squamous papillomas.

***PMLRARA* Transgenic Papillomas Are Not the Consequence of Mutations in Proto-oncogenic *ras* Genes.** Oncogenic ras^{Ki} and ras^{Ha} mutations are the most common reported initiating events in mouse skin papillomas (28). To determine whether activation of ras^{Ha} and ras^{Ki} proto-oncogenes contributed to papilloma formation in *PMLRARA* transgenic mice, ras^{Ha} and ras^{Ki} exons 1 and 2, containing the frequently mutated codons 12, 13, 59, and 61, were sequenced after nested PCR in spontaneously arising *PMLRARA* transgenic papillomas and nontumor-bearing skin as described previously (28). Sequencing of 7 tumors for the ras^{Ha} gene and 3 tumors for the ras^{Ki} gene revealed only wild-type sequence. Thus, in contrast to spontaneous and chemically induced papillomas in nontransgenic mice, papilloma development in *PMLRARA* transgenic mice does not frequently involve ras^{Ha} or ras^{Ki} mutations. This suggests that expression of *PMLRARA* in mouse skin may serve as an alternative initiating pathway for tumorigenesis.

C57/Bl6 Mice Are Readily Susceptible to *PMLRARA*-driven Papilloma Formation. Additional evidence that the mechanism of *PMLRARA* induced papillomagenesis was different from previously characterized mouse skin tumorigenesis models was discovered on crossing the *PMLRARA* transgene onto the skin tumor-resistant C57/BL6 strain. Mice from two *PMLRARA* transgenic lines (506 and 556) were crossed to inbred C57/BL6 mice to minimize the impact of the skin lesions on the analysis of the hematopoietic phenotype. The crosses were only followed for two generations, but the progeny

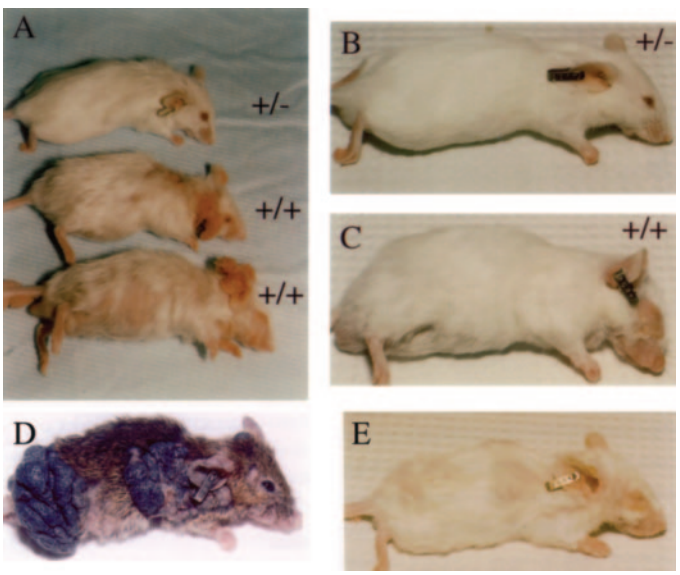

Fig. 4. The severity of the *PMLRARA* transgenic skin phenotype correlates with the level of transgene expression. Hemizygous transgenic mice of line 553 (*A*) and line 565 (*B* and *C*) sustained a less severe skin phenotype (*A–C*) compared with mice homozygous for the transgene (*A* and *C*). The papilloma phenotype was more severe in 8-week-old hemizygous *PMLRARA* transgenic mice of a FVB/N × C57/Bl6J background (*D*) compared with 8-week-old hemizygous *PMLRARA* transgenic mice on an FVB/N genetic background (*E*).

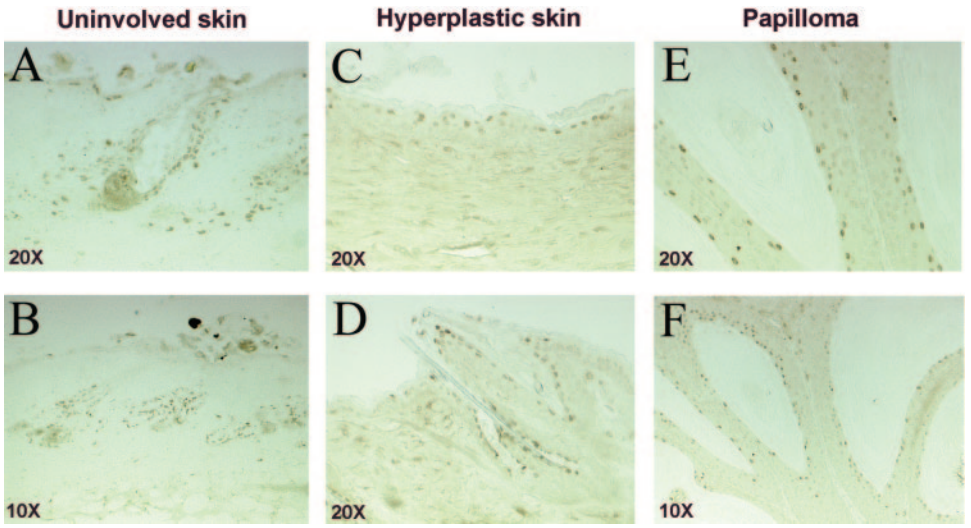

Fig. 5. The *PMLRARA* transgene was expressed in the hair follicles of hMRP-8 transgenic mice. Immunohistochemistry using human-specific PML antibody recognizing the transgenic PML-RARα protein but not endogenous mouse PML in *PML-RARA* transgenic noninvolved skin (*A* and *B*), hyperplastic transgenic skin (*C* and *D*), and a spontaneously developing papilloma from a *PMLRARA* transgenic mouse (*E* and *F*).

rapidly developed substantial skin lesions (Fig. 4; data not shown), which made them impractical to maintain. The histology of the lesions resembled those seen in the inbred FVB/N background (data not shown). This result suggested that tumor induction by the *PMLRARA* transgene occurred through a novel mechanism unrelated to the strain-dependent tumor susceptibility documented previously by other investigators (37–39).

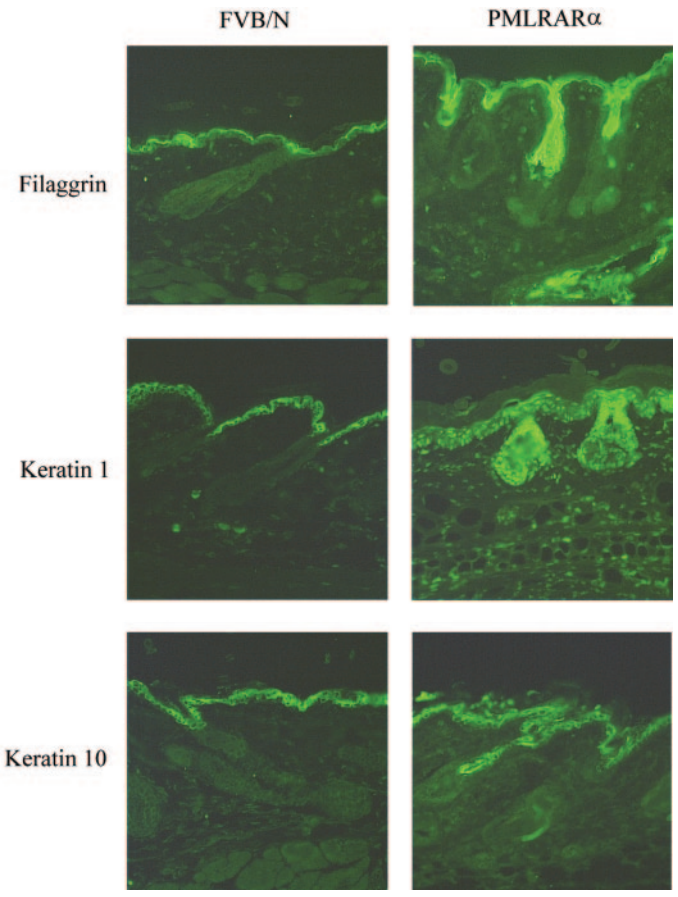

Fig. 6. Epidermoid-type differentiation was detected in *PMLRARA* transgenic hair follicles. Immunofluorescence staining for markers of epidermal differentiation filaggrin (*top panels*), keratin 1 (*middle panels*), and keratin 10 (*bottom panels*) was performed on sections of skin removed from 8-week-old *PMLRARA* (*right panels*) and nontransgenic FVB/N mice (*left panels*).

Administration of ATRA Prevents Papilloma Development. The hyperkeratotic, scaly skin and periorbital edema and reddening of the *PMLRARA* transgenic mice were consistent with the appearance of retinoid deficiency (35, 36). To determine whether administration of ATRA could prevent the skin phenotype of the *PMLRARA* mice, transgenic mice were treated with placebo or 5-mg ATRA pellets implanted in each of 3 15-day-old pups of line 565, before the development of cutaneous papillomas. Three weeks after the pellets were implanted, the animals that received the placebo developed skin changes in the characteristic pattern, but the others had not (see eye reddening and scaly snout phenotype in Fig. 7). Therefore, RA was able to inhibit the development of the skin phenotype in the *MRP8-PMLRARA* transgenic mice. RA normalized the altered hematopoiesis of *MRP8-PMLRARA* transgenic mice as well (9). Implantation of ATRA pellets also abrogated skin tumor formation for both spontaneous and wound-induced tumors (including intentionally wounding by applying a surgical wound clip).

***PMLRARA* Transgenic Mice Exhibit Altered Metabolism of Retinoids.** The similarity of the skin phenotype to vitamin A deficiency and the response to pharmacological ATRA prompted us to examine retinoid metabolism in the *PMLRARA* transgenic mice. Liver levels of retinyl palmitate in *PMLRARA* transgenic and control mice were analyzed by high performance liquid chromatography. Although there was a 25% reduction in the level of retinyl palmitate between transgenic and age-matched control livers, the difference was not statistically significant (Table 1). However, the ability to synthesize RA from retinal was reduced by 24% in liver extracts of transgenic mice (Table 1). An even greater decrease (37%) was found when the assay was conducted in the absence of exogenous retinal (Table 1). Both of these changes are statistically significant. These data suggest that a decrease in endogenous retinal as well as a decreased ability to convert retinal to RA may contribute to the retinoid-deficient phenotype of *MRP8-PMLRARA* transgenic mice. No measurable RA was detected in the absence of NAD (data not shown). These data suggest that decreased endogenous retinal may have been partly responsible for the difference in RA production between the two genotypes. Attempts to measure retinoid metabolism in the skin were not successful.

Skin from *PMLRARA* Mice Has Reduced Retinoid Receptors. Components of the retinoid signaling pathway were evaluated in extracts of nonlesional skin samples from 2 control and 2 transgenic mice. A clear reduction of >75% in both RARα and RXRα was detected in transgenic skin (Fig. 8). Using an antibody that recognizes

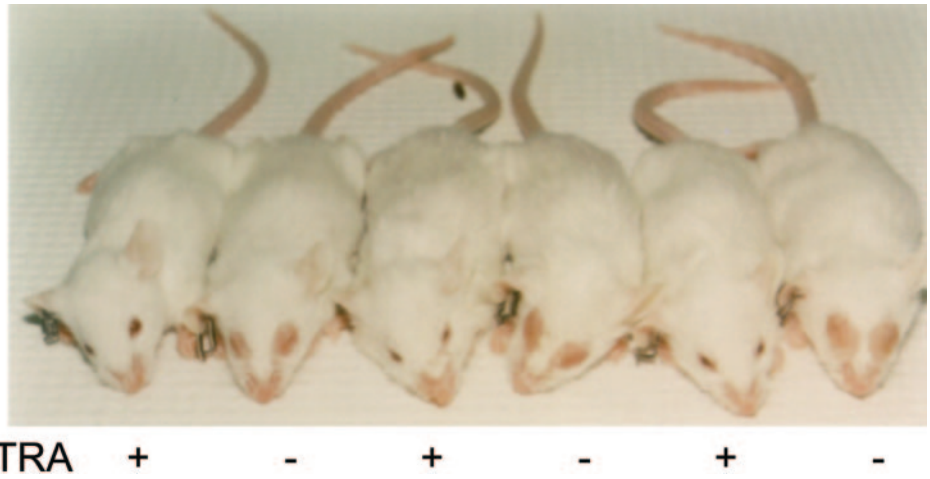

Fig. 7. ATRA pellets prevent the skin phenotype of *PMLRARA* transgenic mice. Six homozygous littermates of line 565 received s.c. 21-day timed-release 5 mg ATRA or placebo pellets when they were 15 days old. Photographs show the mice at 6 weeks of age. Those that received ATRA pellets (+) did not develop the cutaneous manifestations characteristic of this line, whereas those that received placebo pellets (−) developed periorbital edema, erythema, and scaling.

Table 1 *RA synthesis is decreased in PMLRARA* transgenic nonleukemic mouse liver

	Genotype[a]	
	FVBN	*PMLRARα*
Amount of RA produced in 3 h (pmol/ng protein) without exogenous retinal. (n = 4; total of 24 assays for FVB/N, and 20 assays for *PMLRARα*	3.3 ± 1.10	1.96 ± 0.71[b] (37% decrease)
Amount of RA produced in 3 h (pmol/ng protein) with exogenous retinal added. (n = 4; total 33 assays for FVB/N, and 30 assays for *PMLRARα*)	3.23 ± 1.24	2.44 ± 0.90[b] (24% decrease)
Liver retinyl palmitate concentration (μg/g liver), n = 4.	1206 ± 278	909 ± 197 (25% decrease)

[a] Values are reported as mean ± SD with the % of FVB/N control in parenthesis.
[b] Value is significantly different from the corresponding FVB/N control using a two-way ANOVA test, where $P < 0.05$.

both human RARα and PML-RARα (Fig. 8, *left top panel*), the transgene is clearly detected in the skin lysates. Endogenous PML levels are not consistently altered in mice of either genotype.

The *PMLRARA* Transgenic Skin Phenotype Is Independent of the Hematopoietic Phenotype. To determine whether papilloma development was linked to the hematopoietic phenotype, we transplanted transgenic bone marrow cells to nontransgenic hosts and monitored the skin phenotype. Most recipients of bone marrow transplants developed the hematopoietic phenotype, acute myeloid leukemia, including anemia and thrombocytopenia in the absence of increased numbers of WBCs, pale bone marrow, hepatosplenomegaly, and lymphadenopathy. Morphologically and by fluorescence-activated cell sorter analysis (including low level expression of Gr-1 and Mac-1 antigens, characteristic of APL cells; Ref. 40) the leukemic cells were identical to leukemias that arose in nontransplanted *MRP8 PMLRARA* transgenic bone marrow. However, cutaneous papillomas or other features of the skin phenotype did not develop (data not shown). Thus, the papillomas appeared to represent the result of expression of the *PMLRARA* transgene in the skin and other organs of the mice. To address this issue, keratinocytes from *PMLRARA* mice were grafted to athymic nude hosts, and the resulting skin grafts were monitored for phenotypic alterations or tumor formation. Although the grafting procedure creates a wound environment, grafted transgenic and nontransgenic keratinocytes formed an identical integument, and papillomas did not occur (Fig. 9). When v-*ras*^{Ha} was introduced into transgenic and control keratinocytes through retroviral

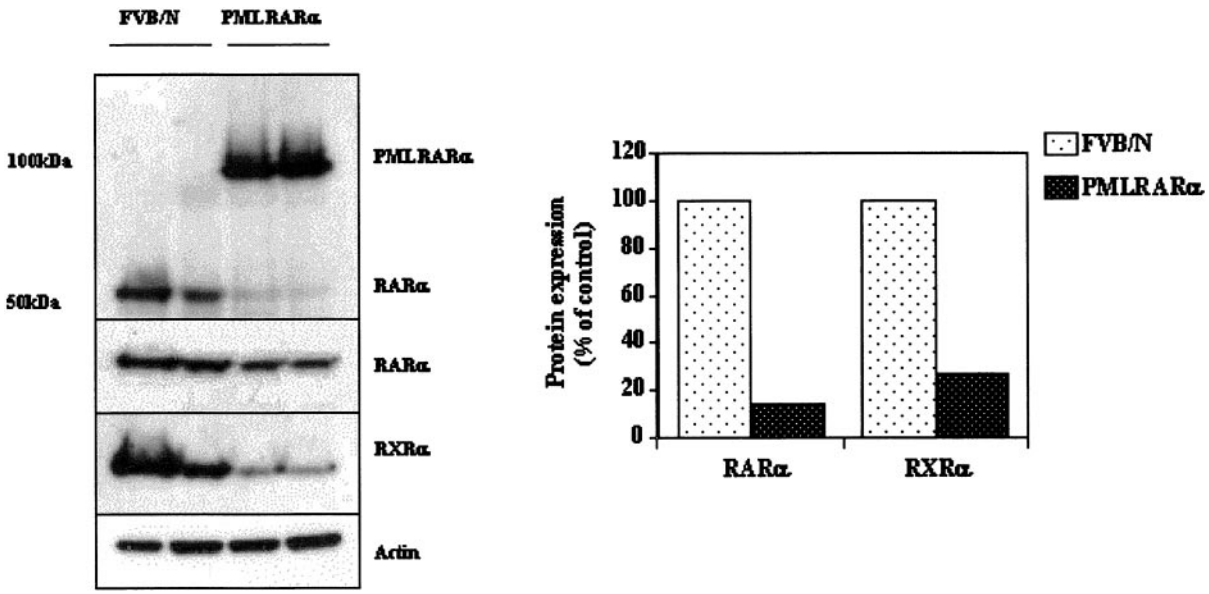

Fig. 8. Nonlesional skin extracts from 2 control FVB/N and 2 transgenic mice were independently assayed for RARα and RXRα protein levels by immunoblotting (*left panel*). The *top left panel* probe was antibody RPα F-115 (29), which recognizes human RARα and PMLRARα (30). The source of the other antibodies is described in "Materials and Methods." The *right panel* quantitates relative expression of RARα and RXRα corrected for actin levels in the same sample.

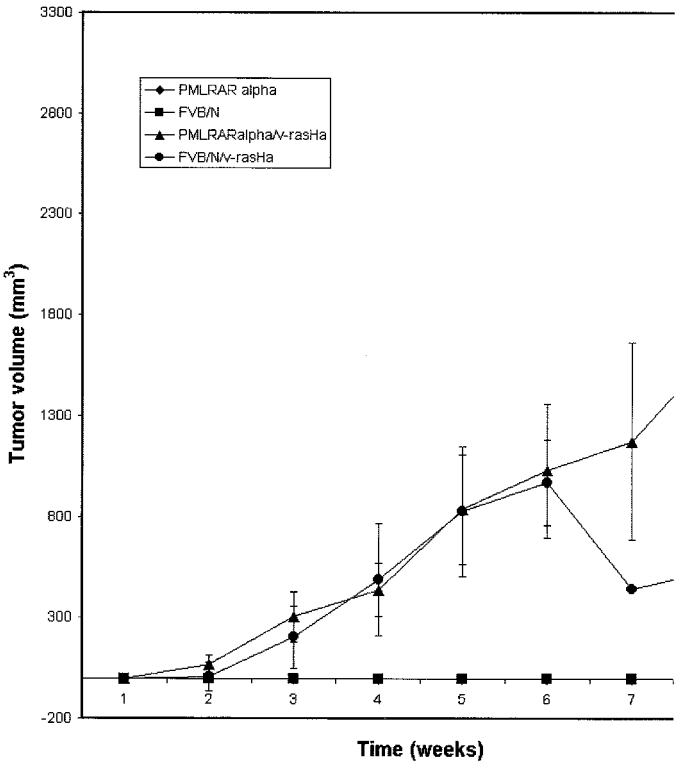

Fig. 9. *PMLRARA* and nontransgenic control keratinocytes do not develop papillomas when grafted to athymic nude mouse hosts. Keratinocytes from transgenic and nontransgenic newborn mice (PML-RARα and FVB/N, respectively) were grafted together with nontransgenic dermal fibroblasts onto athymic nude mice as described in the "Materials and Methods." Additional populations of newborn transgenic and nontransgenic keratinocytes (PML-RARα/v-*ras*^{Ha} and FVB/N/v-*ras*^{Ha}, respectively) were infected with a v-*ras*^{Ha}-expressing retrovirus in culture before grafting with nontransgenic fibroblasts. Tumor volume was measured weekly and plotted as shown.

transduction in culture and recipient cells grafted to nude mice, papillomas did develop in grafts, and the kinetics of tumor growth and tumor sizes in the two genotypes were similar (Fig. 9). Thus, the presence of the transgene did not interfere with *ras*-mediated tumor formation, supporting the notion that the absence of *ras* mutations in transgenic papillomas indicates an alternative pathway in skin carcinogenesis rather than exclusion of a *ras* pathway. These results also suggest that papilloma development in intact *PMLRARA* transgenic mice requires the combined effect of the *PMLRARA* transgene expression in the skin together with a systemic retinoid deficiency.

DISCUSSION

Skin is an important target for retinoid activity. Expression of a wide variety of genes that control keratinocyte proliferation and differentiation are regulated by retinoids (41). In rodent models, retinoid deficiency causes hyperkeratosis with prominent follicular keratinization forming horn plugs, a mottled coat, facial erythema and alopecia, and keratitis (35, 36), changes observed in the *MRP8-PMLRARA* mice. Whereas several other transgenic mouse models for APL involve targeted expression of PML-RARα to bone marrow cells (4, 7, 8), this is the first to exhibit a skin phenotype, extra hematopoietic tumors, or evidence of systemic retinoid deficiency. A likely explanation for this unique result is the use of the *MRP8* promoter to drive transgene expression. MRP8 was detected previously in differentiating skin keratinocytes (19) consistent with transgene expression detected in the granular layer of hyperplastic epidermis and papillomas. This fortuitous targeting and the tissue-specific consequences revealed a unique pathway for cutaneous tumorigenesis.

In *MRP8-PMLRARA* mice, the expression of the PML-RARα fusion protein induced a retinoid-deficient cutaneous phenotype accompanied by papillomagenesis. Transgene expression in epidermal and hair follicle keratinocytes was necessary for the phenotype; lethally irradiated nontransgenic mice reconstituted with *MRP8-PMLRARA* transgenic bone marrow did not exhibit abnormal skin. Because PML-RARα is able to act as a double dominant-negative protein, expression of the transgene in skin almost certainly impaired the function of both PML and retinoid receptors in cells able to give rise to papillomas. However, local effects of the transgene in mouse skin were not sufficient to be tumorigenic, because transplantation of *MRP8-PMLRARA* keratinocytes together with nontransgenic dermal fibroblasts did not produce papillomas or an abnormal skin phenotype at the graft site in athymic nude mouse hosts. This suggests that a systemic effect of transgene expression, in concert with local effects, was required for papilloma development. Transgenic mice displayed phenotypic manifestations of systemic retinoid deficiency as well as biochemical evidence for local interruption of cutaneous retinoid pathways. The systemic manifestations included the skin and eye phenotype, reduced RA synthesis in the liver, and tumor regression by systemic administration of ATRA. Locally, there was a marked reduction in cutaneous levels of RARα and RXRα. These changes have been associated with cutaneous tumors previously (23, 42).

A number of studies have documented that retinoids can inhibit skin papilloma formation induced by application of 7,12-dimethylbenz(*a*)anthracene as an initiating agent and 12-*O*-tetradecanoylphorbol-13-acetate as a promoting agent (43, 44). Retinoids can also be effective as inhibitors of UV light-induced human cutaneous cancers (45), and retinoid deficiency has been identified as a human skin cancer risk factor (45). As stated previously, RARs are reproducibly down-modulated in both human and mouse skin tumors (22, 23), and activation of the *ras* oncogene can result in down-modulation of RARs (42). However, we could not detect *ras* activation in the tumors derived from *MRP8-PMLRARA* mice. Both reduced levels of retinoid receptors and direct interference in transcriptional activity of RARs by the PMLRARα fusion protein are likely to have contributed to the cutaneous phenotype and tumor formation in this model because the severity of the cutaneous phenotype correlated with the level of transgene expression. Furthermore, targeted ablation of RXRα in mouse skin produced severe alterations of hair follicle morphogenesis resulting in epidermoid cysts, alopecia, and overlying epidermal hyperplasia (24). Thus, local interference with hormone receptor signaling can produce a skin phenotype similar to that seen in our study where substantial down-regulation of RXRα levels was detected in the skin of *MRP8-PMLRARA* transgenic mice. Nevertheless, it appears that this local effect must be coupled with a systemic deficiency in vitamin A levels as indicated by the grafting studies.

RA, the active form of vitamin A, is produced from retinol through two oxidation steps that use retinal as the immediate precursor for RA synthesis (1). Retinyl esters represent the storage form of retinol and are hydrolyzed to maintain the homeostatic retinoid physiology. Retinyl esters are derived from dietary retinol and/or carotenoids, and are stored in the liver (reviewed in Ref. 46). The reduced retinyl ester levels and diminished metabolic production of RA in the liver could, in part, reflect an activity of the transgene expressed in the liver perhaps through an effect of circulating myeloid cells. For example, circulating granulocytes that express PML-RARA might influence hepatocytes as they pass through the liver. In addition, MRP8 is expressed focally in the mouse fetal liver (18) in the hematopoietic population, and an influence of transgene expression on the subsequent metabolic activity in adult hepatocytes is conceivable. Altogether, our results indicate that PML-RARα-induced skin tumorigenesis appears to proceed through a novel mechanism involving retinoid

insufficiency. In contrast to the skin phenotype, leukemia was independent of systemic retinoid deficiency as demonstrated by the development of leukemia in nontransgenic recipients of transgenic bone marrow.

The *PMLRARA* transgene appears to act as a conditional initiating factor in skin tumorigenesis, because papillomas form at the site of wounds and evolve rapidly. Similar rapid onset of hepatic tumors in a background of induced hyperplasia was detected when the *PMLRARA* transgene was expressed at high levels in the liver under the control of the metallothionein promoter, supporting a more general oncogenic activity associated with suppression of PML and RAR pathways (6). Our studies indicate that transgene expression is predominant in the more differentiated compartments of hyperplastic and neoplastic skin lesions. These results raise an interesting puzzle as to how an oncogenic factor expressed predominantly in differentiated cells can induce tumors. This example is not unique in experimental skin carcinogenesis. Targeting of oncogenic *ras* to the suprabasal differentiating compartment of the epidermis with a keratin 1 or keratin 10 promoter was papillomagenic previously, although these tumors rarely, if ever, progressed to carcinoma (47, 48). In another study, cutaneous suprabasal expression of c-myc driven by the involucrin promoter reactivated the cell cycle in postmitotic suprabasal cells producing a severe hyperplasia and papillomatous lesions (49). Considering the role for PML in cell cycle control through the Rb pathway and apoptosis through the p53 or DAXX pathways (50), disruption by PML-RARα could recapitulate the biological action of c-myc overexpression. Such a possibility is supported by evidence that the PML-RARα protein can interfere with the transcriptional repression activity of the tumor suppressor Mad (51). Recent evidence for PML involvement in telomere maintenance and DNA methylation (52) provide other potential targets to contribute to cutaneous cancer induction. It should be noted that PML has been implicated as a modifier of experimental cutaneous carcinogenesis, because PML null mice are more susceptible to skin tumor induction than control mice when treated with 7,12-dimethylbenz(*a*)anthracene and 12-*O*-tetradecanoylphorbol-13-acetate (16).

Our findings may have important implications for understanding the development and treatment of human skin tumors. Epidemiological evidence suggests that systemic retinoid status can have a substantial influence on UV-induced human squamous cell cancers (45). This model could contribute to the identification of novel pathways that act in concert with retinoid deficiency in cutaneous oncogenesis. More broadly it has become clear that interactions between neoplastic epithelial cells and abnormalities of non-neoplastic stroma are important in tumor development, maintenance, and progression. The novel observation that retinoid deficiency combines with abnormal gene expression in skin to generate cutaneous papillomas raises the possibility that such systemic/local interactions may be similarly important in the initiation or progression of a variety of human cancers.

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A *PMLRARA* Transgene Results in a Retinoid-deficient Phenotype Associated with Enhanced Susceptibility to Skin Tumorigenesis

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