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The Human Promyelocytic Leukemia Protein is a Tumor Suppressor for Murine Skin Carcinogenesis

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Abstract

Expression of the PMLRAR α fusion dominant-negative oncogene in the epidermis of transgenic mice resulted in spontaneous skin tumors attributed to changes in both the PML and RAR pathways [1]. To determine the contribution of PML to skin tumor susceptibility, transgenic mice were generated on an FVB/N background, that overexpressed the human PML protein in epidermis and hair follicles under the control of the bovine keratin 5 promoter. PML was highly expressed in the epidermis and hair follicles of these mice and was also increased in cultured keratinocytes where it was confined to nuclear bodies. While an overt skin phenotype was not detected in young transgenic mice, expression of keratin 10 (K10) was increased in epidermis and hair follicles and cultured keratinocytes. As mice aged, they exhibited extensive alopecia that was accentuated on the C57BL/6J background. Following skin tumor induction with 7, 12-dimethylbenz[a]anthracene (DMBA) as initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as promoter, papilloma multiplicity and size were decreased in the transgenic mice by 35%, and the conversion of papillomas to carcinomas was delayed. Cultured transgenic keratinocytes underwent premature senescence and upregulated transcripts for p16 and Rb but not p19 and p53. Together, these changes suggest that PML participates in regulating the growth and differentiation of keratinocytes that likely influence its activity as a suppressor for tumor development.

Keywords

transgenic mice; tumor suppressor proteins; nuclear bodies; cytokeratin-10; senescence; keratinocytes

INTRODUCTION

A remarkable insight emerging from the molecular era of carcinogenesis research is the universality of certain molecular pathways in the pathogenesis of a diverse array of tumor types. Although particular tissues require specific programs for control of growth and differentiation, neoplastic evolution frequently involves a common set of genes and their downstream effectors. Some of the common pathways were first identified from rare tumor

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This article is dedicated to the late Adam J. Berry, who contributed youthful excitement, technical expertise and dedication to this and other studies in the Laboratory of Cellular Carcinogenesis and Tumor Promotion.

types or syndromes such as retinoblastoma, ataxia telangiectasia, xeroderma pigmentosum and Li Fraumeni syndrome where genetics provided an experimental approach to unravel the molecular etiology. Acute promyelocytic leukemia (APL) provided another such opportunity when it was discovered that a reciprocal chromosomal translocation fusing the RARA and PML genes was the underlying cause of the differentiation defect that formed the basis for promyelocytic leukocytosis [2]. The prevailing evidence suggests that the highly expressed fusion protein usurps the function of both PML and RAR to interrupt the normal pathway of myelocytic maturation. Promyelocytic Leukemia (PML), a phosphoprotein localized to specific nuclear bodies [3] is now recognized to participate in multiple cell activities including the control of growth, senescence and apoptosis.

Since the initial discovery of the PML gene through studies of the fusion protein in APL, evidence has accumulated that PML participates in cancer pathogenesis in other target sites. This conclusion comes from three types of studies. (1) Examination of human tumors for the expression of PML indicates a frequent loss of expression, particularly in later stages of progression [4], [5]. (2) Reconstitution of bladder, liver, prostate, mammary, renal and Hela tumor cells with exogenous PML suppresses growth or induces apoptosis *in vitro* or inhibits tumor formation when the cells are grafted *in vivo* [6], [7], [8], [9], [10], [11], [12], [13]. (3) Reduction to heterozygosity of the normal PML allele enhances leukemia in patients and in an animal model of PML-RARA leukemia [14]. While these studies support a tumor suppressor function for PML, the issue has not been addressed in an animal model of multistage tumor development where the presence of excessive PML in the normal target tissue can affect tumor formation.

Several studies have suggested indirectly that PML can protect against tumor development on mouse skin treated with chemical carcinogens. Ablation of PML enhances susceptibility of mice to chemically induced skin carcinogenesis [15]. Targeting the PML-RARA fusion gene to the epidermis induces spontaneous skin papillomas and carcinomas with unusual properties [1]. Tumors form in the absence of *ras* gene mutations, and C57BL/6J mice, normally resistant to skin carcinogenesis, are more susceptible than FVB/N mice. From these observations and others, it was concluded that cutaneous expression of the oncogenic fusion transgene produced a systemic Vitamin A deficiency and abrogation of keratinocyte PML function produced an environment that encouraged spontaneous skin tumor development. To date there are no data to address the function of PML directly in skin. It is known that epidermal PML increases in chronic graft vs host disease, and this is associated with foci of apoptosis [16]. However, PML is increased in psoriatic epidermis, a situation where proliferation is increased [4]. In order to establish the contribution of PML to skin function and carcinogenesis independently of RAR α function, transgenic mice were generated that overexpress PML under the control of the keratin 5 (K5) promoter, thus restricting cutaneous expression to epidermis and hair follicles.

MATERIALS AND METHODS

Generation of transgenic animals

Transgenic mice were generated by cloning hPML (PML-1 M73778) from the pCMX expression vector kindly donated by Dr. R. Evans [17], [18] into the 3' end of the β -globin intron in a construct containing the bovine keratin 5 promoter, the rabbit β -globin intron and an SV40 Poly(A) termination signal [19]. The construct was linearized with *Sall* and *NotI*, and a 9.8 Kb fragment was electroeluted from agarose and microinjected into fertilized oocytes from the inbred FVB/N strain [20]. The presence of the transgene was assessed by Southern blotting with standard protocols [21] with a full length PML probe prepared by digestion of pcDNA/PML with *NotI*. This fragment was purified from agarose and labeled with ³²P. Genotyping in more than 20 tail samples for each one of 6 founder lines and their

progeny was done by PCR with primer pairs to hPML, Poly A, B-globin and K5. Primers for PML were PML #1 (5'-TCGGAGGAGGAGTTCCAGTTTC-3') PML #2 (5'-TCGGCAGTAGATGCTGGTCAG-3'). Primers for β globin were β globF (5'-TGCATATAAATTCTGGCTGGCG-3'), β globR (5'-GCATGAACATGGTTAGCAGAGGG-3'), primers for K5 promoter were K5F1 (5'-TGCGTCTTTCTTTCTCTCAA-3') and K5R1 (5'-TTTCAGAAACACGCCTGGAGA-3'); primers for polyA were polyA F2 (5'-TGCGACTCCTGCATTAGGAA) and polyA R2 (5'-ACATCACCGATGGGGAAGAT-3'). To study strain dependence, transgenic K5-PML FVB/N mice were crossed to C57BL/6J males or females for 5 generations. Adult animals of the F1 to F3 offspring were observed weekly over a period of a year.

Chemical carcinogenesis

Groups of 15 FVB/N strain mice at 4 months of age had their dorsal hair trimmed and were initiated by one topical application of 100 μ g DMBA. Starting a week later all mice were treated weekly by topical application of 5 μ g of TPA for up to 20 weeks. [22]. The number of tumors was determined weekly. Promotion was stopped at week 22 post initiation, and mice were observed weekly to quantify papilloma and carcinoma incidence. Tumors were collected, measured in three dimensions by calipers and processed for histology. Hematoxylin-eosin stained tumor sections were assessed to establish tumor phenotype. Percent of mice bearing tumors (incidence), average number of tumors per mouse (yield) and malignant conversion time, were calculated and plotted using GraphPad Prism, GraphPad Software, San Diego, CA, Statistical differences were also calculated with GraphPad Prism.

Keratinocyte culture

Transgenic and wild type keratinocytes were prepared from newborn littermates according to [23]. Cells were typically cultured for 4–5 days in 0.05 mM calcium containing SMEM medium (Invitrogen Carlsbad, CA), unless specified and were used to prepare cell lysates or for immunohistochemistry. Some of the experiments were done with newborn keratinocytes from the K5-PMLx C57BL/6J F3.

Senescence assays

Primary keratinocytes were cultured as above for up to ten days. Senescence was assayed according to the method by [24], which detects β -galactosidase at pH 6, upon senescence in culture. Briefly, cells that had been cultured for different times were fixed in 5% glutaraldehyde in PBS, pH 7.2, for 5 minutes and washed twice with PBS pH 7.2, containing 1 mM MgCl_2 . Then 500 μ l of staining solution (0.12 mM $\text{K}_3\text{Fe}[\text{CN}]_5$, 0.12 mM $\text{K}_4\text{Fe}[\text{CN}]_6$, 1 mM MgCl_2 in PBS pH 6.0 containing 1 mM X-gal) was added per well and the cells were incubated for 24–48 hours at 37 °C in a bacterial incubator.

Real Time PCR

For real-time PCR analysis, the expression level of p16, p19, Rb and p53 cDNAs was determined using a BioRad iQ iCycler and Gene Expression Macro (version 2.0) from Biorad. cDNA (diluted 1:200 in the final volume reaction) was measured from triplicates samples using iQ SYBR Green Supermix (Biorad). The primer sequences were as follows: GAPDH, CCT GCA CCA CCA ACT GCT TA and TCA TGA GCC CTT ACA ATG; Rb, CCT TGC ATG GCT TTC AGA TTC ACC and CCT TCT CCA TCC TTG GAC TGC TTA; mouse p19ARF CCG CAC CGG AAT CCT G and TGA GCA GAA GAG CTG CTA CGT G; mouse p53 (NM_011640) CAG CTT TGA GGT TCG TGT TTG T and ATG CTC TTC TTT TTT GCG GAA A; p16primers were previously described [25]. Relative standard curves were generated from log input (serial dilutions of pooled cDNA) versus the threshold

cycle (Ct). The slope of the standard curve was used to determine the efficiency of target amplification (E) using the equation $E = (10^{-1/\text{slope}} - 1) \times 100$. Relative quantitation was used to calculate the $2^{-(\Delta\Delta Ct)}$ formula where $\Delta\Delta Ct$ represents the difference corrected for GAPDH used as internal control.

Cell adhesion assays

Cytomatrix cell adhesion strips pre-coated with individual matrix proteins (laminin, fibronectin, collagen I, collagen IV and vitronectin) (Chemicon, Temecula, CA) were used according to manufacturer's instructions. Fibronectin-collagen (human-bovine) was prepared according to [26] in complete medium containing 1.4 mM Ca^{2+} and 20 mM HEPES and contained 1% w/v human fibronectin (BD Biosciences, San Diego, CA), 1% Vitrogen 100 collagen (type I bovine snout collagen) Collagen Corporation, and 10 % BSA. For adhesion assays, keratinocytes were allowed to adhere to the different matrix or to uncoated tissue culture plastic for times varying between 30 min and 8 h in duplicate or triplicate wells of 24 well plates. Wells were washed twice gently with PBS containing Ca^{2+} and Mg^{2+} , stained with 0.2% crystal violet in 10% EtOH for 5 min and rinsed 3 times with PBS containing Ca^{2+} and Mg^{2+} . The dye was solubilized from adhered cells by a 50/50 mixture of 0.1 M NaH_2PO_4 , pH 4.5 and 50% EtOH for 5 minutes. Absorbance of 4 repeats for each well was measured at 540 nm in a microplate reader.

Tissue histology, immunohistochemistry and immunofluorescence

Dorsal skins from transgenic or wild type littermates were fixed in 70% ethanol overnight, processed and embedded in paraffin. Six-micron sections were deparaffinized and antigen retrieval was performed with 0.01% trypsin and 0.05 % saponin. Antibodies used were anti keratins 1, 5, 10 and 14 (Covance, Berkeley, CA) directly conjugated to FITC, filaggrin, (Covance) at 1:500; anti PML H238 polyclonal antibody (Santa Cruz, CA). PCNA staining was done according to the method by [27] with monoclonal anti-PCNA 19A2 (Coulter Immunology, Hialeah, FL). Secondary antibodies were biotinylated IgG F(ab)₂ fragment from Jackson with subsequent ABC kit detection (Vector Laboratories, Burlingame, CA) followed by hematoxylin counterstain (Fisher Scientific Co.) or fluorescent Alexa 468 or 568 (Molecular Probes, Eugene, OR) followed by mounting in DAPI containing mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence was detected with a Zeiss LSM 510 confocal microscope. Negative controls for all experiments replaced the primary antibody with dilution buffer.

Immunoblotting

Cell lysates were prepared with 0.25 M Tris HCl buffer pH 6.8, containing 20% β -mercaptoethanol and 5% SDS. Proteins were electrophoretically transferred from NuPage gels (Invitrogen, Carlsbad, CA) onto PVDF membranes (Immobilon-P, Millipore). PML (Santa Cruz, antibody H238) was used at 1:500 dilution followed by anti-rabbit IgG horseradish peroxidase linked (Amersham Pharmacia). For detection, chemiluminescent substrates were from Pierce Chemical.

Immunofluorescence staining of cultured keratinocytes

Cells were grown for 4 days on coverslips that had been precoated with fibronectin/collagen when indicated or in two well glass culture chambers such as LabTek II (Nalge Nunc Int., Naperville, IL), with medium changes every other day. Cells were fixed with 50/50 v/v methanol/acetone for one minute, washed with PBS and blocked with PBS containing 3% BSA. Fluorescent conjugated antibodies to keratins 10 and 14 were used at 1:1000 dilution, anti PML (H238) was used at 1:500 dilution, p16 (M156, Santa Cruz) used at 1:500 dilution.

RESULTS

Generation of transgenic mice and expression of the transgene

A plasmid was constructed encoding human PML-1 gene [17] (and now referred to as PML VI, [28]) under the regulation of the bovine keratin 5 promoter (Fig. 1A). In postnatal mice this promoter activity is restricted to dividing basal layer keratinocytes of the epidermis, outer root sheath and some other stratified epithelia such as oral and tongue [19]. Southern blots identified integration sites in multiple founders (Fig. 1B), with several showing amplification of the insert (e.g. H4 and J1). A PCR approach was optimized for genotyping, and more than 20 tail samples for each of 6 founder lines and their progeny were tested for hPML, Poly A, B-globin and K5. Only offspring of the H4 line showed positive bands for all primer pairs (Fig. 1C).

Northern blotting of all six lines suggested that only 3 lines (H4, G6 and A3) were expressing the transgene (data not shown), and these lines were further investigated for transgene expression. Transgenic protein expression was detected by immunohistochemistry in the skin of mice from 3 lines (G6, A3 and H4) and additionally by immunofluorescence and by immunoblots in primary cultured keratinocytes from the H4 line (Fig. 2, A, B, C). In cultured transgenic keratinocytes, PML was increased but remained largely in characteristic nuclear bodies whereas in skin *in vivo* PML was both nuclear and cytoplasmic in epidermis and hair follicles. Expression of the transgenic protein was also found in some tissues from an animal of the A3 line (kidney, tongue, esophagus and testes) (data not shown). Out of these three positive lines, only one line (H4) was able to breed and yield progeny beyond F3, a finding that was not explored further. All experiments reported here were performed with the K5-PML H4 line, which will subsequently be referred to as K5-PML mice.

K5-PML mice, and to a much lesser extent wild type littermates, developed alopecia with aging. In the FVB/N background this was seen relatively late in life (> 8 months). However, when the transgene was bred onto the C57BL/6J background (referred to as K5-PMLb, Fig. 3A), alopecia occurred in mice as young as two months. By 11 months nearly half of the K5-PMLb mice had hair loss. Histologically, components of the pilosebaceous apparatus such as sebaceous glands and deep follicles were present, but hair was not being produced (Fig. 3B). Some follicles produced keratin pearls as shown in Fig. 3B (arrow). At the initiation of the first hair cycle, K10 expression was increased in transgenic skin relative to wildtype and extended into the anaphase follicles where it is not normally expressed (Fig. 3C). Furthermore, expression of K10 was increased in cultured transgenic keratinocytes in both low calcium medium and in response to calcium induced differentiation (Fig. 3D). While it is not clear by what mechanism alopecia occurred, immunostaining of adult skin in areas of alopecia indicated that keratin 10 staining was prominent in the hair follicle infundibulum of transgenic mice (Fig. 3E) suggesting abnormal differentiation in these follicles. We could not detect specific upregulation of K1 or filaggrin suggesting a specific effect on K10 in K5-PML epidermis (not shown). At the initiation of the first hair cycle, the proliferative potential of K5-PML basal epidermis was also reduced compared to wild type epidermis as detected by PCNA labeling in the skin of young mice (Fig. 3F). While these differences were consistent, they did not reach statistical significance.

Cutaneous chemical carcinogenesis is suppressed by PML overexpression

Tumors were induced in groups of 4 month old K5-PML and FVB/N littermates by topical application of one dose of 7,12-dimethylbenz[a]anthracene (DMBA) followed by weekly applications of 12-O-tetradecanoylphorbol-13-acetate (TPA). Papilloma development was delayed slightly in that a smaller percentage of transgenic mice developed tumors until week 15 (Fig. 4A). Furthermore, peak tumor multiplicity was decreased by 35% in transgenic

mice, and this was observed throughout the tumor induction period suggesting this was not due to tumor regression (Fig. 4A). However the final percent of mice with tumors at 22 weeks was not different among the groups (Fig. 4A). There was also a reduction in mean papilloma size by about 30% in the K5-PML mice (Fig 4B). Papillomas converted to carcinomas with the same frequency in transgenic and wild type mice, but the time to the clinical appearance of carcinomas was delayed by about 5 weeks in K5-PML mice (Fig. 4C). This could reflect an overall reduction in papillomas at risk, the delay in appearance of benign lesions or a true effect on malignant conversion. Carcinomas developing on transgenic mice were smaller than those on wild type mice (Fig. 4B) suggesting that PML does affect carcinoma growth. These data suggest that elevation of PML in the target tissue for skin carcinogenesis has a suppressive influence on the rate of tumor development, tumor growth and conversion of tumors from benign to malignant but not on the overall risk of developing a tumor. The proliferative response to TPA influences the rate of tumor development in the mouse skin model [29]. To detect this response, a single dose of TPA was applied on the back of transgenic and wild type animals, and 48 hours later the skin was examined for PCNA labeling. The average skin PCNA labeling index was lower (32%) for transgenic animals than the index for wild type mice (47%) suggesting that PML expression reduces the proliferative response mediated through PKC signaling.

Cultured K5-PML keratinocytes are prone to senescence

PML has been associated with premature senescence in other cell types [30], [31], [32]. Strikingly, there was a 5–7 fold increase in senescing keratinocytes from K5-PML epidermis versus wild type epidermis after 10 days in culture when measured by SA- β Gal staining (Fig. 5A, B). This change in a senescence associated biochemical marker was associated with cell spreading and flattening in cultured transgenic keratinocytes, a phenotypic marker of senescence in keratinocytes (Fig. 5B). We also examined the ability of wild type and transgenic keratinocytes to attach to various matrix proteins. While the results indicate a trend for K5-PML keratinocytes to attach more avidly than wild type keratinocytes to tissue culture plastic and collagen I and IV, the differences were not significant, except in the case of adhesion to tissue culture plastic (not shown). Together these results indicate that upregulation of PML in keratinocytes alters intrinsic properties related to differentiation, adhesion and senescence. Such changes could contribute to the reductions seen in tumor number, growth and time for malignant conversion. However, we were not able to detect an increase in SA- β Gal staining in papillomas from either genotype (data not shown).

Expression of several growth inhibitory genes is increased by PML

PML is known to influence the expression of specific genes [33]. Among these p16 is a protein known to be associated with senescence. Transgenic keratinocytes express cytoplasmic p16 after one day in culture, and p16 is prominently seen in senescing cells by day nine (Fig. 5C). We could not detect Rb in these same cultures. Transcripts for both p16 and Rb were modestly increased in transgenic keratinocytes by day 6 of culture (Fig. 6). This increase precedes the accumulation of senescent cells as seen in Fig. 5. In contrast p53 and p19 transcripts were not elevated in transgenic keratinocytes during the culture period examined.

DISCUSSION

The construction of mice with a skin targeted PML transgene and testing for sensitivity to skin tumor induction has provided additional support for the previously proposed link between PML expression and tumor suppression [34]. Previous evidence for this connection comes from *in vitro* analysis of PML modified tumor cell lines, or tumor grafts of PML modified cells or tumor induction in hosts with constitutive ablation of the PML gene. We

now show that upregulation of PML expression can prolong the latency for tumor eruption, reduce the final tumor burden and tumor size, and extend the time for premalignant to malignant conversion in a multistage inducible tumor model. Our results further indicate however, that PML does not prevent malignant conversion. Since malignant conversion in this model is strongly linked to genomic instability [35], the results do not support the suggestion that the tumor suppressive effects of PML are through genetic stability mechanisms [34], [33]. A number of mechanisms have been proposed to explain the putative tumor suppressor function of PML [34], [33], [30]. The most examined among these is the regulation of senescence, and several intersecting pathways have been proposed to mediate this PML function. In several cell types, PML regulates both the transcription and activation of p53 and participates in p53- mediated senescence and apoptosis [30], [31], [32]. However, p53 is a strong inhibitor of malignant conversion in the mouse skin model, and p53 loss does not increase the yield of papillomas [36], [37]. One might expect PML overexpression to inhibit malignant conversion and not alter the frequency of benign tumors if it were functioning through a p53 pathway. Our qPCR data indicate that p53 transcripts are not elevated in the cultured keratinocytes overexpressing PML further suggesting that PML mediated skin tumor suppression is working through a different pathway.

Of particular interest is a previous report that heterozygosity of p63 upregulates PML and p16 contributing to premature senescence in cultured mouse keratinocytes. Furthermore, p16 and PML were co-upregulated in senescing epidermis *in vivo* [38]. In that study, suppression of PML by shRNA abrogates the senescence phenotype [38]. Our study directly confirms that PML accelerates keratinocyte senescence *in vitro* and further suggests that the upregulation of p16 might be a consequence of PML action in keratinocytes. Early alopecia and epidermalization of hair follicles are consistent with premature cutaneous senescence and premature skin aging was reported to be associated with PML upregulation [38]. A similar phenotype was observed as a consequence of stem cell depletion when telomerase is ablated [39]. The alopecia we observed is accentuated in C57BL/6J mice compared to mice on the FVB/N background. This is of interest in light of the enhanced sensitivity for spontaneous skin tumor development of C57BL/6J mice expressing a skin targeted PMLRARA transgene that would inhibit PML function in the skin [1]. This suggests that genetic background of mouse strains influences sensitivity to modulation of PML, and C57BL/6J mice may be particularly sensitive to the tumor suppressive and pro-senescence influence of the protein. Together these data support a role for senescence in the tumor suppressive function of PML.

Two other pathways of interest have emerged from analysis of the K5-PML transgenic mice. We have detected an increase in keratin 10 expression in both skin and cultured keratinocytes and an upregulation of transcripts for Rb at day 6 associated with an increase in senescent cultured transgenic keratinocytes. Keratin 10 and the Rb pathway have been connected previously in keratinocytes both *in vitro* and *in vivo*. Introduction of a keratin 10 transgene into human keratinocytes activates the Rb pathway and induces growth arrest [40], [41]. Targeting keratin 10 as a transgene to the basal epidermis of mice causes epidermal hypoplasia and hyperkeratosis through Rb dependent growth inhibition. In this model, skin tumor formation and tumor size were reduced when the transgenic mice were crossed with a skin tumor prone second transgenic mouse line [42]. Our report now suggests that PML may be a missing link in this connection,

This report is the first to expose the consequences of PML upregulation on the epidermis and hair follicles *in vivo*. We have established that PML can be a tumor suppressor protein in this model and can influence keratinocyte biology directly. Our results suggest that keratinocyte senescence and accelerated differentiation may be manifest as premature alopecia *in vivo*. Of particular interest is the finding that genetic background can influence

the consequences of PML action in mice, a finding that must be considered in other mouse models designed to study PML. While we have connected these phenotypic changes to premature senescence, other pathways downstream from PML have not been ruled out. With the ever expanding understanding of PML trafficking, multiple isoforms, protein processing and the role of PML bodies in cellular function, this model can be adapted to explore those pathways and connect them to the interesting biology that PML produces.

Abbreviations

PML	(promyelocytic leukemia)
RAR	(retinoic acid receptor)
DMBA	(7, 12-dimethylbenz[a]anthracene)
TPA	(12-O-tetradecanoylphorbol-13-acetate)
CDK	(cyclin-dependent kinase)
APL	(Acute promyelocytic leukemia)

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Fig. 1.

A. Human PML (PML-1 M73778) was cloned downstream of the K5 regulatory region, into the 3' end of the β -globin intron in a construct containing the bovine keratin 5 promoter, the rabbit β -globin intron and an SV40 Poly(A) termination signal [19]. B. Southern blot analysis of DNA from founder lines I1, J1, H4, H1, and G6. Wt represents a full length PML probe prepared by digestion of pcDNA/PML with NotI. C. Genotyping by PCR of a typical litter of K5-PMLH4, testing consistently positive for all primer sets.

Fig. 2.

A. Immunohistochemistry of paraffin embedded skin sections of mice from line K5-PMLG6 and control FVB/N stained for PML. B. Immunofluorescence of keratinocytes cultured 4 days in medium containing 0.05 mM Ca^{2+} , from line K5-PML H4 and control FVB/N with anti-PML (H238, Santa Cruz). Localization is observed in nucleus (arrow) identified by DAPI staining. C. Immunoblots in primary cultured keratinocytes from the H4 line and control littermates cultured for 4 days in medium containing increasing calcium concentration (0.05–0.2 mM).

Fig. 3. K5-PML mice develop permanent alopecia. **A.** Eleven and sixteen month old male and five month old female K5-PML mice and littermates are shown. Mice were F2 crosses K5-PML (FVB/N) x C57BL/6J. Figures are labeled tg and wt for simplicity. **B.** Hematoxylin-eosin staining of a margin between haired and alopecic skin (40x). Right panel shows a keratinized follicle (arrow) in an alopecic area (200x). **C.** K5-PML H4 transgenic mice express the differentiation marker K10 more extensively than wild type littermates. Ethanol fixed skin sections of wild type and transgenic littermates as newborns and 20 days pp were stained with antibodies to K14 (green) to mark the basal cells, and K10 (red) to visualize differentiated cells. Arrow denotes K10 staining in anagen hair follicles in K5-PML skin. **D.**

Primary keratinocytes grown in medium containing 0.05 mM or 0.2 mM calcium were immunostained to detect K10 expression and nuclei were labeled with DAPI. E. Skin sections from two K5-PML and FVB/N littermates were immunostained for K10 showing areas of alopecia with extension of K10 expression into the hair follicle in the transgenic skin. F. Percent PCNA positive epidermal cells in skin during the first hair cycle. Basal interfollicular cells and those in the upper portion of the hair canal were counted excluding the dermal papilla area. Five random fields were scored for each section.

Fig. 4. Induction of skin tumors by chemical carcinogens. A. Fifteen mice per group were treated once with DMBA, followed by weekly applications of TPA. A. (left) papilloma incidence (percent tumor-bearing mice) counted between weeks 10 and 22 post-DMBA treatment, and (right) papilloma multiplicity (number of papillomas per mouse), was calculated for each time point. By two way ANOVA, the difference between both curves is significant ($p < 0.0001$) in the case of papilloma multiplicity but not in the case of papilloma incidence. B. Papilloma and carcinoma sizes (mm³) were measured in remaining tumors upon termination of the experiment. Scatter plots represent all tumor sizes and their mean. T test of the mean values was not significant for papillomas and $p = 0.04$ for carcinoma sizes. C.

Conversion to carcinoma was delayed approximately 5 weeks in K5-PML transgenic mice. Log-rank test of the differences between groups was significant at $p=0.04$.

Fig. 5.

A. Keratinocytes from K5-PML newborn mice senesce after 9 days in culture. Total and SA-β-galactosidase positive cells [24] were scored. Two random fields were scored per well in a 24 well plate and the experiment was repeated twice. *** indicates $p=0.01$. B. Top, representative images of SA-β-gal staining of wild type and transgenic keratinocytes of animals of the FVB/N background at 10 days in culture. Bottom panels show phase contrast morphology of keratinocytes from K5-PML or wildtype littermates of the C57BL6/J background cultured for 9 days. C. Cultures of wildtype and transgenic keratinocytes were immunostained for p16 (green) and DAPI (blue) at day 1 and day 9 in culture.

Fig. 6. Quantitative real time PCR analysis of keratinocyte transcripts. K5-PML and FVB/N keratinocytes were isolated and cultured for indicated times prior to RNA isolation. Quantitative PCR was performed as described in Materials and Methods and presented relative to the GAPDH control. The experiments were repeated two times on independent cultures isolated from different mouse litters.