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Expression of a PMLRAR[alpha] transgene in mice

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Expression of a PMLRARα Transgene in Mice

by

Diane Elizabeth Brown

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Abstract

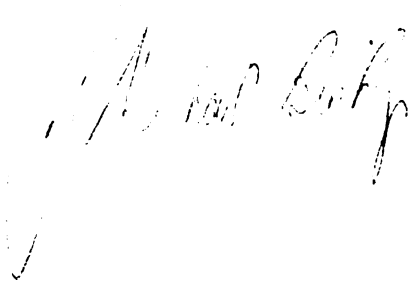
The malignant cells of acute promyelocytic leukemia (APL) contain a reciprocal chromosomal translocation that fuses the *PML* and*RARα* genes. In order to test the hypothesis that the chimera *PMLRARα* plays a role in leukemogenesis, we used an*MRP8* promoter to express a *PMLRARα* transgene in the myeloid cells of mice.

PMLRARα transgenic mice exhibited impaired neutrophil maturation, and some progressed to overt APL. Both the preleukemic state and the leukemia could be transplanted to nontransgenic mice, and the transplanted preleukemia could progress to APL. The APL recapitulated several clinical features of the human disease, including a characteristic response to retinoic acid. Retinoic acid caused the leukemic cells to differentiate *in vitro* and *in vivo*, eliciting remissions of both the preleukemic state and APL in mice.

Human *MRP8* expression is not limited to myeloid cells; Mrp8 has also been documented in epithelial tissues, including the epidermis, and the *hMRP8-PMLRARα* mice displayed other phenotypes consistent with transgene expression in non-myeloid cells. The *PMLRARα* mice developed epidermal abnormalities ranging from subtle changes in hair follicles to large papillomas, at least one of which progressed to malignancy. We have not yet determined whether epidermal differentiation was impaired analogously to neutrophilic differentiation, but there was an analogous sensitivity to retinoic acid, which inhibited the development of the epidermal lesions. The *PMLRARα* mice were also prone to keratinizing squamous metaplasia of their preputial and clitoral glands; a similar phenomenon in the mammary glands might explain why the females sometimes had difficulty nursing their pups. A few lymphoid malignancies have also been observed: a B-cell tumor

led to both leukemic meningitis and an ascending paralysis suggestive of a paraneoplastic disorder.

Our results suggest that *PMLRARα* can impair the differentiation of cells in diverse tissues and initiate the development of several types of tumors. Analysis of the murine APLs may reveal some of the additional events that cooperate with *PMLRARα* expression in leukemogenesis, increasing our understanding of leukemogenesis by *PMLRARα* in particular and tumor progression in general. The mice also provide a model for preclinical testing of novel therapeutic strategies against APL.



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Malabattan-Weissman not only ordered my supplies, but her wonderful questions and her art reminded me of the beauty in our work.

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My parents, family and friends helped my sanity by reminding me that there is life outside the lab, which on many late nights and early mornings seemed very remote indeed. In their own way, so did my four-legged friends, who provided a gentle distraction when it was desperately needed.

And last, but in no way least, I thank Craig Schmid, whose friendship, and encouragement, and symphony tickets provided a needed anchor through the best and worst of times.

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Chapter 1:
Introduction

The growth, differentiation, and death of many cells must be closely coordinated in a multicellular organism. Checks and balances are needed to ensure that developing organs generate the proper complement of cells, and mature tissues renew themselves only as needed. Within individual cells, a tightly woven network of regulatory factors coordinates internal programming with external signals, determining whether cells initiate division, differentiate, or die. Cancers result when this delicate balance is upset in favor of proliferation, and the cancerous cell propagates itself at the expense of the organism.

In recent years, tremendous progress has been made towards understanding the genetic basis of cancer by unraveling the regulatory networks governing cellular proliferation, differentiation and apoptosis. These networks are generally robust: in most cells, multiple regulatory pathways must be disrupted before uncontrolled growth results. By the time a cancer is detected, many genetic changes may have accumulated. A particular type of change is often seen resulting from translocations between chromosomes: if the translocation breakpoint occurs within introns of genes, it may fuse genes from the two parent chromosomes. These fusion genes may alter the balance between differentiation and proliferation by affecting the function of either parent gene or by novel activity arising in the combined gene. The present study was designed to ask whether a characteristic

translocation fusion gene associated with acute promyelocytic leukemia did, in fact, initiate the development of the disease.

Acute Promyelocytic Leukemia and t(15;17)

Acute promyelocytic leukemia (APL) was first recognized in 1957 as a distinct clinical entity in which accumulation of promyelocytes in bone marrow was accompanied by a rapidly progressive hemorrhagic diathesis (Hillestad 1957). Promyelocytes represent a stage in the differentiation of the neutrophil lineage at which the cells have begun to accumulate the primary but not yet the secondary cytoplasmic granules characteristic of the mature cells (Figure 1.1). Today classified as FAB subtype M3, APL cases comprise approximately 10% of acute myeloid leukemias in adults (Bennett 1985; Stone 1990). APL is not associated with a particular age of onset: while the median age of APL patients at onset is mid-forties, it has been reported in children less than a year old (Gilbert 1987; Knowles 1992). The clinical picture is dominated by the coagulopathy, which is the major cause of morbidity and mortality in the disease (Knowles 1992). The discovery that myeloid leukemia cells could be differentiated *in vitro* with retinoic acid led to differentiation therapy of APL with all-trans retinoic acid, which has considerably improved the clinical picture for APL patients (Breitman 1980; Breitman 1981; Huang 1987b). Before ATRA treatment, up to half of patients died of the coagulopathy during induction chemotherapy (Cordonnier 1985); recent reports suggest that 80-90% of patients achieve complete remission when ATRA is added to

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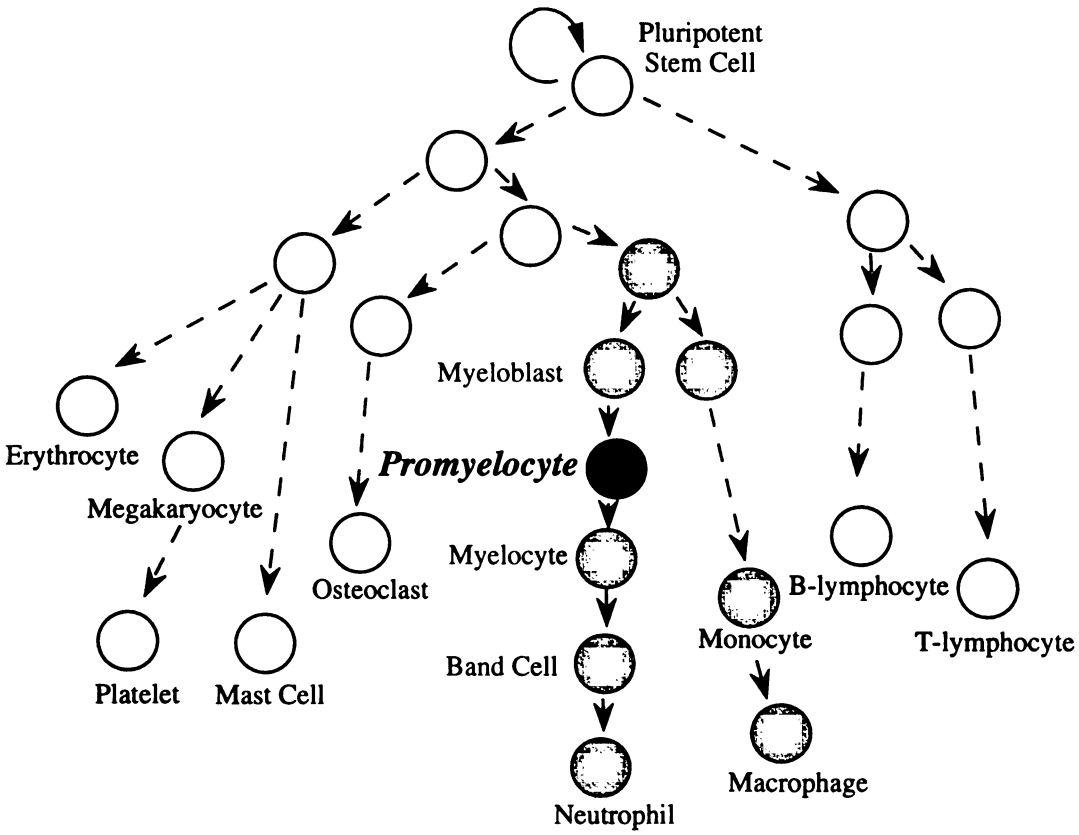
Figure 1.1 Hematopoiesis

The relationships between cells derived from the hematopoietic stem cell are depicted. The granulocyte and monocyte lineages, in which Mrp8 is expressed, are shaded.

Figure 1.1
Hematopoiesis and *hMRP8* expression



=Cell in which *MRP8* should drive transgene expression

traditional cytotoxic chemotherapy, and long term survival rates may surpass 50% (Degos 1994; Fenaux 1996).

The dramatic effects of ATRA differentiation therapy on APL highlight another feature of the disease, the characteristic t(15;17)(q24;q21) translocation (Rowley 1977; Larson 1984; Lo Coco 1992; Grignani 1994). This translocation, which is unique to APL, fuses the *PML* gene on chromosome 15 with the retinoic acid receptor alpha (*RARα*) gene on chromosome 17 (de The 1990; Kakizuka 1991), and ATRA is a ligand for Rarα. In fewer than 1% of APL cases, variant translocations have been seen which fuse other genes to *RARα* (Najfeld 1989; Chen 1993a; Chen 1993b; Corey 1994; Licht 1995; Redner 1996), but as most of those patients did not respond to ATRA, the underlying biology may be somewhat different.

PML

The *PML* gene, discovered through investigation of the t(15;17) translocation (de The 1990; Kakizuka 1991), codes for a protein whose function is unclear. *PML* has homology to a family of genes defined by several zinc finger motifs (the RING finger and B-boxes), which include genes coding for transcription factors and nucleic acid processing proteins (Freemont 1991; Reddy 1991; Reddy 1992); the *PML* protein coding sequence is otherwise unremarkable. In addition to *PML*, two other members of this gene family, *T18* and *RET*, have been found in oncogenic translocations (Miki 1991; Lanzi 1992). Pml itself has not been shown to transactivate transcription directly or to be involved in nucleic acid processing, although it may affect transactivation from a heterologous promoters (Guiochon-Mantel 1995). While *PML* expression is detectable in most tissues and cell lines (Goddard 1991; Goddard 1992),*PML* knockout mice are supposed to be viable,

demonstrating that Pml is not essential for life. Unfortunately, to date there have been no peer-reviewed publications describing the *PML* knockout mice in detail. A recent meeting abstract reported that the *PML* knockout mice were particularly susceptible to a type of infection known as botryomycosis, suggesting that Pml is required for normal immune function; cells from these mice also cloned more readily and grew faster than cells containing *PML*, and the knockout mice were twice as likely to develop tumors following DMBA and TPA treatment as wild type mice, suggesting that Pml has tumor suppressor activity (Wang 1996). Further evidence of an anti-proliferative effect of Pml comes from studies in cultured fibroblasts, in which *PML* expression inhibited proliferation and transformation (Mu 1994; Liu 1995).

The subcellular localization of Pml is also suggestive of a role in control of cellular proliferation, perhaps in response to immunomodulators. Within the cell, Pml shuttles back and forth between the cytoplasm and electron-dense nuclear structures known variously as nuclear bodies or NBs, ND10, Pml oncogenic domains (PODs), or kr bodies (for clarity, I will refer to them as NBs) (Ascoli 1991; Stuurman 1992; Xie 1993; Dyck 1994; Koken 1994; Weis 1994). NBs undergo morphological alteration in synchrony with the cell cycle (Koken 1995; Terris 1995); in response to hormonal or cytokine stimulation (Clark 1978; Fitzgerald 1983; Koken 1995; Korioth 1995; Terris 1995); when inflammation is present (Terris 1995); when cells are treated with organic arsenic (Rivi 1996); and in the presence of viral proteins such as HSV-1 IE Vmw110 (Everett 1994). Viral oncoproteins such as E1a, SV-40 large T antigen and Ebna5, and the murine Int-6 protein, a putative tumor suppressor, also localize to NBs, as does Plzf, the product of a gene found fused to *RARα* by an

11;17 translocation in a few cases of APL (Carvalho 1995; Desbois 1996; Koken 1996; Szekely 1996).

RARα

RARα is a member of the nuclear hormone receptor gene family (Mangelsdorf 1995b). Rarα heterodimerizes with RXR, a promiscuous partner of many nuclear hormone receptors (Mangelsdorf 1995a); in the presence of retinoic acid, Rarα/RXR heterodimers activate transcription from retinoic-acid responsive elements (RAREs). While *RARα* mutations have not been identified in other cancers (Morosetti 1996), another nuclear hormone receptor gene, *TEC*, has been found fused to another gene by a translocation identified in human chondrosarcoma (Labelle 1995).

Studies of vitamin A deficiency have uncovered requirements for retinoids in vertebrate growth and development, and in the maintenance of adult tissues, particularly secretory epithelia (Wolbach 1925; Stuurman 1992; Jansen 1995). Analysis of mice lacking *RAR*s confirm that *Rars* are involved in many aspects of skeletal morphogenesis, development of neural crest derivatives, and visceral organogenesis (Lohnes 1994; Mendelsohn 1994). RAREs are found in the promoter regions of several vertebrate HOX genes, suggesting a pathway through which Rars might mediate these effects (Marshall 1996). Although there is substantial functional redundancy among *RARα, β,* and *γ, RARα* is expressed at higher levels and in more tissues than the others, suggesting that it probably is the major mediator of retinoid signaling (Dolle 1990). Of particular relevance to the present study, retinoids have been shown to play important roles in hematopoiesis and epidermal differentiation.

Atlhough the most prominent hematopoietic defect in retinoid deficiency is anemia (Wolbach 1925), generalized immune impairment is also seen (Sommer 1996), suggesting a role for retinoids in the development of multiple hematopoietic lineages. More recent studies support a broad role for retinoids in hematopoiesis. In vitro, retinoids increase the production of early erythroid and myeloid precursor cells (Koeffler 1985). A similar cell population is identified by expression of a dominant negative *RARα* in primary bone marrow cultures: early stem cell factor-dependent precursor cells proliferate, but are still capable of giving rise to lymphoid, myeloid or erythroid lineage cells if treated with appropriate stimuli (Tsai 1994). In addition to this early block affecting multiple lineages, dominant negative *RARα* further inhibits neutrophilic differentiation at the promyelocyte stage (Tsai 1993). Taken together, these studies imply a need for retinoid-mediated signaling at two distinct stages in hematopoiesis, one in early multilineage precursors, and one specific to the neutrophil lineage.

Skin responds to retinoid treatment with increased proliferation of cells that continue to differentiate appropriately (Jarrett 1980; Rook 1992). In retinoid deficiency, the skin becomes hyperkeratotic (Frazier 1931; Wechsler 1979; Nakjang 1988; Rook 1992). Mice deficient in *RARα* and *RARγ* do not recapitulate this deficiency phenotype, however, suggesting robust functional redundancy among retinoid receptors in the epidermis (Lohnes 1994). Transgenic mice expressing dominant negative *RARα* in skin, which can block the action of Rars α, β and γ (and possibly other RXR heterodimerization partners as well), have hypoplastic skin with inadequate barrier function, suggestive of impaired keratinocyte differentiation (Imakado 1995; Saitou 1995)]. In the epidermis, as in the hematopoietic system,

retinoids increase proliferation of precursor cells and stimulate differentiation of maturing cells.

PMLRARα

PMLRARα retains the bulk of the coding region of both parent genes since the t(15;17) breakpoints cluster in the 3' portion of the *PML* gene and the second intron of the *RARα* gene. Although they lead to slight variations in PmlRarα, the different breakpoints within the *PML* gene are not associated with different biochemical or clinical outcomes (Biondi 1995; Fukutani 1995); the known breakpoints result in nearly all recognized functional motifs of Pml and Rarα being found in PmlRarα rather than RarαPml. RarαPml retains only a minor transactivation activity from RARα and a variable amount of serine-rich sequence from PML. *PMLRARα* is also more consistently expressed than *RARαPML*, being found in virtually all cases of APL, while *RARαPML* mRNA is present in 50-70% of cases.

PmlRarα has a functional Pml dimerization domain, and makes homodimers and heterodimers with Pml (Daniel 1993; Dyck 1994; Weis 1994). PmlRarα disrupts the association between Pml and NBs, dragging Pml into smaller nuclear aggregates, although it is not clear whether this is functionally important (Grignani 1996). Another protein found in NBs, SP100, is also shifted away from them by PmlRarα (Grignani 1996). While we do not know whether these visible changes are key to normal Pml activity, they may indicate a pathway by which PmlRarα could affect cell growth and differentiation.

Like Rarα, PmlRarα is a retinoic-acid dependent transcription factor (de The 1991; Pandolfi 1991). It can bind to RAREs as homodimers or RXR heterodimers, which have distinct binding preferences: PmlRarα

homodimers prefer the same sequences as Rarα homodimers while PmlRarα/RXRa heterodimers prefer the same sequences as Rarα/RXRa heterodimers (Mader 1993; Perez 1993; Jansen 1995). PmlRarα generally acts similarly to Rarα at RAREs (de The 1991; Pandolfi 1991; Kastner 1992), depending on the cellular context, but not always: Rarα acts as an RA-dependent inhibitor on AP-1 dependent promoters, but PmlRarα inhibits those promoters in the absence of RA and activates them in its presence (Doucas 1993). Transglutaminase II, which is upregulated by ATRA in the presence of PmlRarα but not Rarα (Benedetti 1996), may thus represent a class of genes differentially regulated by PmlRarα and Rarα. PmlRarα has also been shown to alter activity at heterologous promoters, augmenting transcription from progesterone responsive promoter in the presence of the progesterone receptor and its ligand, perhaps by binding to the progesterone receptor (Guiochon-Mantel 1995). In several ways, therefore, PmlRarα can affect retinoid signaling via RAREs, other hormone responses via binding to and sequestration of RXR, or alter transcription from heterologous promoters.

PmlRarα has been shown to block the differentiation of a pair of hematopoietic cell lines: U937 monocytic leukemia cells no longer differentiated to neutrophils if stimulated by low doses of retinoic acid and less frequently underwent apoptosis (Grignani 1993); and K562 myeloid leukemia cells no longer differentiated to erythrocytes upon hemin stimulation (Grignani 1995). Further investigations of *PMLRARα* expression in U937 cells demonstrated that the ability to homodimerize and to move SP100 away from NBs correlated with the ability to block differentiation, but that binding to and transactivation from RAREs, movement of Pml away

from NBs, and binding to and sequestration of RXRs were not sufficient to account for PmlRarα effects in these cells.

The potential mechanisms by which PmlRarα could alter proliferation and differentiation of myeloid cells toward APL are plentiful, even without envisioning novel activities generated by the juxtaposition of Pml and Rarα domains. While studies in cultured cells have fruitfully illuminated some of these pathways, they did not answer the question of whether *PMLRARα* expression initiates changes leading to the development of APL.

Evaluation of *PMLRARα* function in transgenic mice

In order to examine whether *PMLRARα* initiates the development of APL, we decided to express a *PMLRARα* cDNA as a transgene in mice. This approach allowed for *PMLRARα* expression in the normal hematopoietic microenvironment of intact bone marrow.

We chose the *hMRP8* promoter cassette to drive transgene expression in myeloid cells (Lagasse 1994). The *hMRP8* cassette was derived from a human genomic clone coding for a calcium binding protein called macrophage migration inhibitory factor related protein (Mrp8) (Burmeister 1986; Zwadlo 1988). *hMRP8* expression has been reported in many tissues, most prominently in neutrophils and monocytes (Dale 1983; Hayward 1986; Dorin 1987; Odink 1987). Interpretation of reported *hMRP8* expression is complicated by the varied terms used to connote either complexes containing Mrp8 or Mrp8 itself: some papers refer to cystic fibrosis antigen or L1 antigen, both of which pertain to complexes of Mrp8 and Mrp14 (Andersson 1988a; Andersson 1988b; Bruggen 1988); to Cp10 or calgranulin A or W2 antigen, which pertain to Mrp8 itself (Wilkinson 1988); Mac387, a monoclonal antibody, recognizes an Mrp14 epitope, and Mrp14 is typically found together

complexed with Mrp8 (Brandtzaeg 1988); and some use the current nomenclature of Mrp8. Outside of the myeloid lineage, expression has been reported in many different epithelial tissues, especially in the presence of inflammation (Gabrielsen 1986; Odink 1987; Wilkinson 1988; Zwadlo 1988; Tang 1993; Wistow 1993; Fanjul 1995; Lugering 1995). At the time that this project was undertaken, transgene expression from the *hMRP8* cassette had been documented only within the myeloid lineage (Lagasse 1994).

Overview of results

We generated transgenic mice bearing either a *PMLRARα* or an *RARαPML* cDNA under the control of the *hMRP8* promoter. The *hMRP8-RARαPML* mice appeared normal; however, as we did not verify transgene expression in these mice, we draw no conclusions about the effect of *RARαPML* on hematopoiesis.

We have noted two major consequences of transgene expression in the *hMRP8-PMLRARα* mice. First, in the hematopoietic system, neutrophil differentiation was impaired and some of the mice progressed to acute promyelocytic leukemia; these results are the subject of chapters two and three. Second, they had hyperplastic epidermis which was prone to develop papillomas at sites of wounding, and at least one of these papillomas progressed to malignancy; these results are discussed in chapter four.

We have noted several other abnormalities in the mice, which have not yet been intensively investigated; because they may provide additional insights into PmlRarα activity or have an impact on the maintenance of the animals, keratinizing squamous metaplasia, a respiratory distress syndrome, and lymphoid malignancies are discussed in chapter five.

Chapter six summarizes and presents some conclusions derived from the results presented. The appendices include additional materials and methods, and summary tables regarding phenotypes observed, survival, and causes of death in selected transgenic lines.

Chapter 2:

A *PMLRARα* Transgene Initiates Murine Acute Promyelocytic Leukemia

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Abstract

The malignant cells of acute promyelocytic leukemia (APL) contain a reciprocal chromosomal translocation that fuses the genes PML and RARα. In order to test the hypothesis that the chimera PMLRARα plays a role in leukemogenesis, we expressed a PMLRARα cDNA in myeloid cells of transgenic mice. PMLRARα transgenic mice exhibited impaired neutrophil maturation early in life, which progressed at a low frequency over the course of several months to overt APL. Both the preleukemic state and the leukemia could be transplanted to nontransgenic mice, and the transplanted preleukemia could progress to APL. The APL recapitulated several clinical features of the human disease, including a bleeding diathesis and a response to retinoic acid. Retinoic acid caused the leukemic cells to differentiate in vitro and in vivo, eliciting remissions of both the preleukemic state and APL in mice. Our results demonstrate that PMLRARα impairs neutrophil differentiation and initiates the development of APL. The transgenic mice described here provide an apparently accurate model for human APL that includes clear evidence of tumor progression. The model should be useful for exploring the molecular pathogenesis of APL and the mechanisms of the therapeutic response to retinoic acid, as well as for preclinical studies of therapeutic regimens.

Introduction

The malignant cells of acute promyelocytic leukemia (APL) carry a reciprocal chromosomal translocation, t(15;17)(q24;q21) (Rowley 1977; Warrell 1993; Grignani 1994). The translocation fuses two genes (de The 1990; Kakizuka 1991): *RARα*, which encodes the retinoic acid receptor alpha (Rarα); and *PML*, which encodes Pml, a nuclear phosphoprotein of unknown function (Chang 1995). Prior to the identification of the genes at the t(15;17) breakpoint, all-trans retinoic acid (ATRA) had been shown to induce complete remission in APL patients by differentiation of the leukemic cells (Huang 1987a; Warrell 1991). This convergence of a novel therapeutic strategy and the molecular analysis of the translocation focused interest on how the resulting *PMLRARα* and *RARαPML* fusion genes functioned in the pathogenesis and therapeutic response of APL.

The *PMLRARα* fusion encodes the bulk of both gene products (Figure 2.1a). PmlRarα retains activities of both Pml and Rarα, including transcriptional activation in response to retinoic acid, and can inhibit differentiation of cultured cells (de The 1991; Kakizuka 1991; Pandolfi 1991; Kastner 1992; Daniel 1993; Grignani 1993; Perez 1993; Dyck 1994; Rousselot 1994; Weis 1994; Grignani 1995). In contrast, the coding domain of the reciprocal *RARαPML* fusion contains little of note (Figure 2.1b) and has not been shown to have any phenotypic effects. While *PMLRARα* is expressed in

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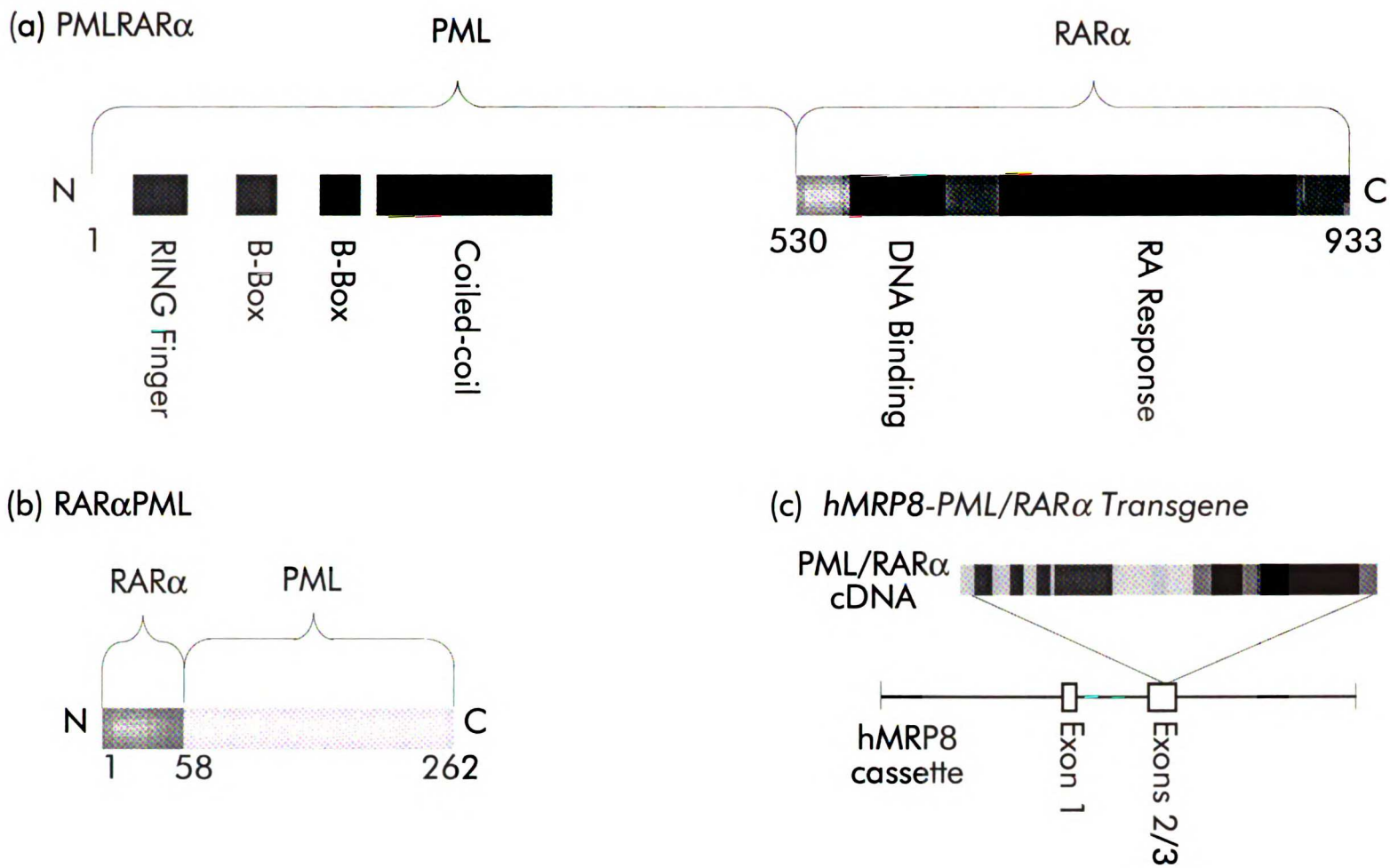
Figure 2.1 T(15;17) fusion genes and the *hMRP8-PMLRARα* transgene

(a) *PMLRARα* and (b) *RARαPML* are drawn to scale. Known sequence motifs are highlighted. (c) The cloning site in the *hMRP8* vector replaces the protein coding sequence of the *hMRP8* genomic clone.

Figure 2.1 *PMLRARα, RARαPML*, and the *hMRP8-PMLRARα* transgene



virtually all cases of APL, *RARαPML* is frequently not expressed (Borrow 1992). For these reasons it has been assumed that *PMLRARα* is responsible for the initiation of leukemogenesis, but this view has not been directly authenticated.

Here we report the establishment of a transgenic mouse model that documents the ability of *PMLRARα* to initiate leukemogenesis. The mice develop two apparently unrelated abnormalities. The first is a severe papillomatosis of the skin that we will describe elsewhere. The second is a disturbance of hematopoiesis that presented as a partial block of differentiation in the neutrophil lineage of the transgenic mice and then progressed at low frequency to overt APL. The leukemias appear to be a faithful reproduction of the human disease, including a therapeutic response to ATRA that reflects differentiation of the leukemic cells. Both the preleukemic state and the overt leukemia can be transplanted into nontransgenic hosts. The mouse model described here can be used to explore the pathogenesis and treatment of APL.

Materials and Methods

Generation of transgenic mice

A human *PMLRARα* cDNA (Pandolfi 1991) (whose chromosome 15 breakpoint lies in breakpoint cluster region 1 as defined in Pandolfi 1992 (Pandolfi 1992)), was cloned into the *hMRP8* expression cassette (Lagasse 1994). β-galactosidase was also cloned into the same cassette. Injection fragments were purified from agarose gels, filtered through Elutips (Schleicher & Schuell, Keene, NH), and resuspended in 10 mM Tris pH 8/0.1 mM EDTA at 2.5 ng/ml.

Transgenic animals were prepared and maintained in accord with UCSF animal care guidelines. Single-cell embryos were isolated from inbred

FVB/N mice (Taketo 1991), and microinjections and embryo transfers followed standard procedures (Hogan 1994). In some cases *hMRP8-LacZ* was coinjected with *hMRP8-PMLRARα*, but the presence of *hMRP8-LacZ* was superfluous to the results presented here. Tail DNA was prepared by the method of Laird et. al. (Laird 1991) for analysis by Southern blot or PCR.

Isolation of cells from tissues

Blood was obtained from anesthetized animals by venipuncture of the retro-orbital venous plexus. Bone marrow was obtained by flushing HBSS through mouse long bones, followed by filtering through nylon mesh. Spleen or lymph node suspensions were prepared in HBSS and filtered through nylon mesh. When necessary, red cells were lysed with ACK lysing buffer (Coligan 1991).

Blood smears and bone marrow smears were prepared according to standard hematological techniques. For cytospins, up to 1 x 10^5 cells were prepared in a Cytospin® 2 (Shandon Lipshaw, Pittsburgh, PA) at 400 rpm for 10 minutes or 1000 rpm for 3 minutes.

Immunofluorescence

Cytospins prepared as above were fixed for 5 minutes in -20°C methanol and air dried. Samples were rehydrated for 1 minute in PBS, blocked for 15 minutes in PBS with 10% normal goat serum (PBS/NGS), and incubated for one hour with rabbit polyclonal antiserum prepared against a GST-Pml fusion protein diluted 1:4000 in PBS/NGS (the antiserum was a kind gift of Dr. K. S. Chang, M.D. Anderson Cancer Center, University of Texas, Houston, TX). Washing with PBS/NGS was followed by 30 minutes of incubation with goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) at 1:400 in PBS/NGS. The slides were then washed sequentially with PBS/NGS,

PBS, and water before mounting in Prolong medium (Molecular Probes, Eugene, OR) plus 1 μM DAPI.

Staining of cytospins and aspirates

Slides were stained with Wright's Giemsa and Azure B dyes (Curtis Mattheson Scientific, Houston, TX) or Sudan Black B stain (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's directions.

Fluorescence-activated cell sorting

Single cell suspensions prepared as above were stained with the indicated antibodies and analyzed on a FACScan Flow Cytometer (Becton Dickinson & Co., Mountain View, CA). Cells were incubated with monoclonal antibodies for 20 to 30 minutes on ice, then allowed to settle through a calf serum cushion to remove unbound antibodies. Cells were resuspended in staining medium (HBSS with 2% FCS) containing propidium iodide (PI) at 4 mg/ml. At least 10,000 cells were analyzed per sample. Dead cells that stained with PI were gated out at the time of analysis. FACS data were analyzed with the CellQuest program (Becton Dickinson). Anti-Gr1-FITC and anti-MAC1-PE were obtained from PharMingen, San Diego, CA.

Methylcellulose cultures of bone marrow

5×10^4 viable bone marrow cells per 35 mm tissue culture dish were cultured in duplicate in Methocult M3230 methylcellulose medium (StemCell Technologies, Vancouver, BC) supplemented with 2% PWM Spleen-Conditioned Media (StemCell Technologies) with or without 1 μM ATRA (Sigma). Cultures were examined at 7 days. Cells isolated from the cultures were counted and stained as above.

Western blotting

Western blotting was performed as described (Robbins 1995) with rabbit polyclonal antiserum raised against a glutathione S-transferase fusion protein encompassing amino acids 420 to 462 of the human Rarα protein (Gaub 1992). Before use, the antiserum was affinity purified with the analogous maltose binding protein fusion.

Histological preparations

Tissues were formalin fixed, embedded in paraffin, and sections were stained with hematoxylin and eosin for histological examination.

Transplantation of leukemia

10^7 viable cells isolated from bone marrow, spleen or lymph nodes of animals with leukemia were resuspended in 200 µL of HBSS and injected into the tail veins of 5-8 week old FVB/N mice.

Transplantation of bone marrow

Total bone marrow isolated from the tibiae and femurs of a single donor was divided for intravenous injection into six recipient mice. 5-12 week old FVB/N mice were prepared for transplantation by cesium irradiation totaling 10.5 Gray, divided in two doses 3-6 hours apart.

ATRA treatment

At the indicated times after transplantation, ATRA was administered to mice via one of two routes: intraperitoneal injection of 100 µL of ATRA (Sigma) or ethanol vehicle suspended in canola or peanut oil at 54 mg/kg/day or subcutaneous implantation of a 21-day-release pellet containing 5 mg ATRA or placebo (Innovative Research of America, Sarasota, FL).

Results

Expression of *PMLRARα* in transgenic mice

In order to examine the effect of *PMLRARα* on granulocyte development, we created mice that expressed a human *PMLRARα* cDNA (Pandolfi 1991) from the *hMRP8* promoter cassette (Figure 2.1c), which drives transgene expression in myeloid cells (Lagasse 1994) . Injections into FVB/N embryos yielded nine transgenic lines.

Levels of *PMLRARα* transgene expression in the bone marrow of transgenic mice varied from virtually undetectable to several fold higher than NB4 human promyelocytic leukemia cells (de The 1990; Lanotte 1991) (Figure 2.2a). To determine which cells in the transgenic bone marrow expressed *PMLRARα*, bone marrow cells or peripheral blood leukocytes were analyzed by immunofluorescence using antiserum directed against human

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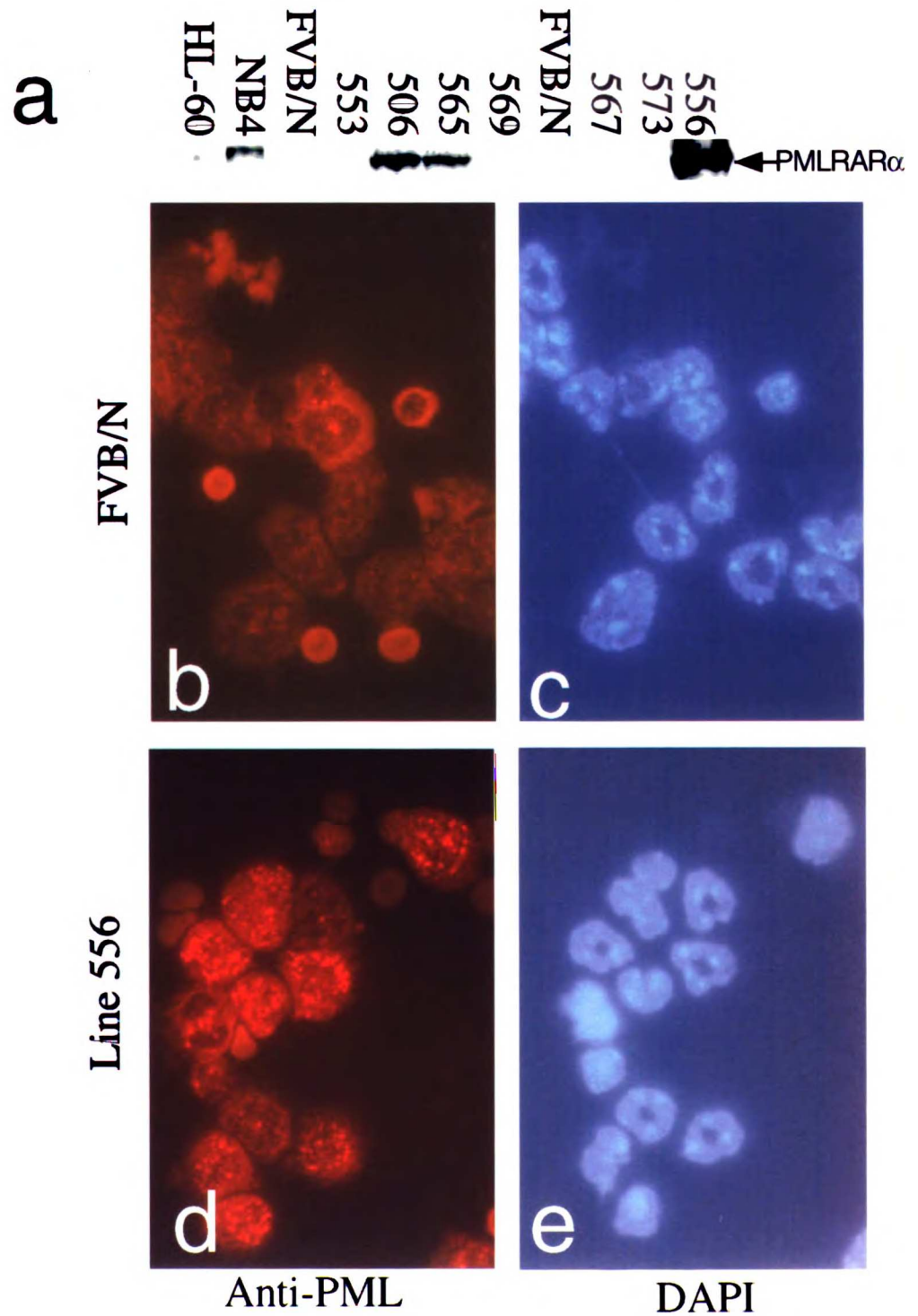
Figure 2.2 *PMLRARα* expression in bone marrow of transgenic mice

(a) Western blot. Total cell lysates from equal numbers of unfractionated bone marrow cells from the indicated *hMRP8-PMLRARα* transgenic lines or control cell lines were loaded into each lane. The blot was probed with antibodies against human Rarα. HL-60 human myeloid leukemia cells do not contain t(15;17) (Dalton 1988). NB4 human APL cells (Lanotte 1991) express PMLRARα (de The 1990). FVB/N samples were from non-transgenic control mice.

(b-e) Immunofluorescence. Bone marrow cells from healthy 8-week old mice were cytospun and incubated with antiserum specific for human Pml (b,d) and the DNA-intercalating dye DAPI (c,e). (b,c) Control FVB/N. (d,e) Transgenic line 556. Original magnification 250X.

Figure 2.2
Expression of PMLRARα
in transgenic bone marrow



Pml. PmlRarα was distributed in a speckled nuclear pattern consistent with previous reports (Kastner 1992; Daniel 1993) (Figure 2.2d). Simultaneous staining with DAPI highlighted the distinctive indented, horseshoe- or doughnut-shaped nuclei characteristic of murine granulocytes (Figure 2.2e), verifying that the transgene was expressed in the granulocyte lineage. In lines where transgene expression was undetectable by western blotting, very few cells were stained by the anti-Pml antibody; in lines with high levels of expression, many more of the cells were stained (data not shown). Altogether, we detected PmlRarα expression in granulocytes from eight of nine transgenic lines.

Neutrophil maturation is impaired in *PMLRARα* transgenic mice

When examined microscopically, the peripheral blood and bone marrow of *PMLRARα* transgenic mice resembled the controls (data not shown). Differential cell counts did not show any substantial differences. However, although neutrophilic cells from transgenic mice appeared morphologically normal, when we subjected cells isolated from peripheral blood or bone marrow to fluorescence-activated cell sorting (FACS) using markers for myeloid differentiation, subtle aberrations were apparent. Within each transgenic line, the aberrations were consistent in all mice examined regardless of age. The transgenic samples displayed lower levels of the Gr-1

antigen (Fleming 1993) (Figure 2.3a-c), expression of which increases as neutrophils mature (Hestdal 1991). The transgenic samples also had lower than normal levels of side-scatter (Figure 2.3d-f), reflecting a low cytoplasmic granularity, which is typical of immature granulocytes. Both side-scatter and Gr-1 expression were inversely related to the level of transgene expression. In contrast, levels of CD11b, a cell-surface marker that also increases with myeloid differentiation, were not diminished (data not shown). Granulocytic cells from the transgenic mice therefore combined features of both immature and mature cells. These data reveal that *PMLRARα* blocks some aspects of

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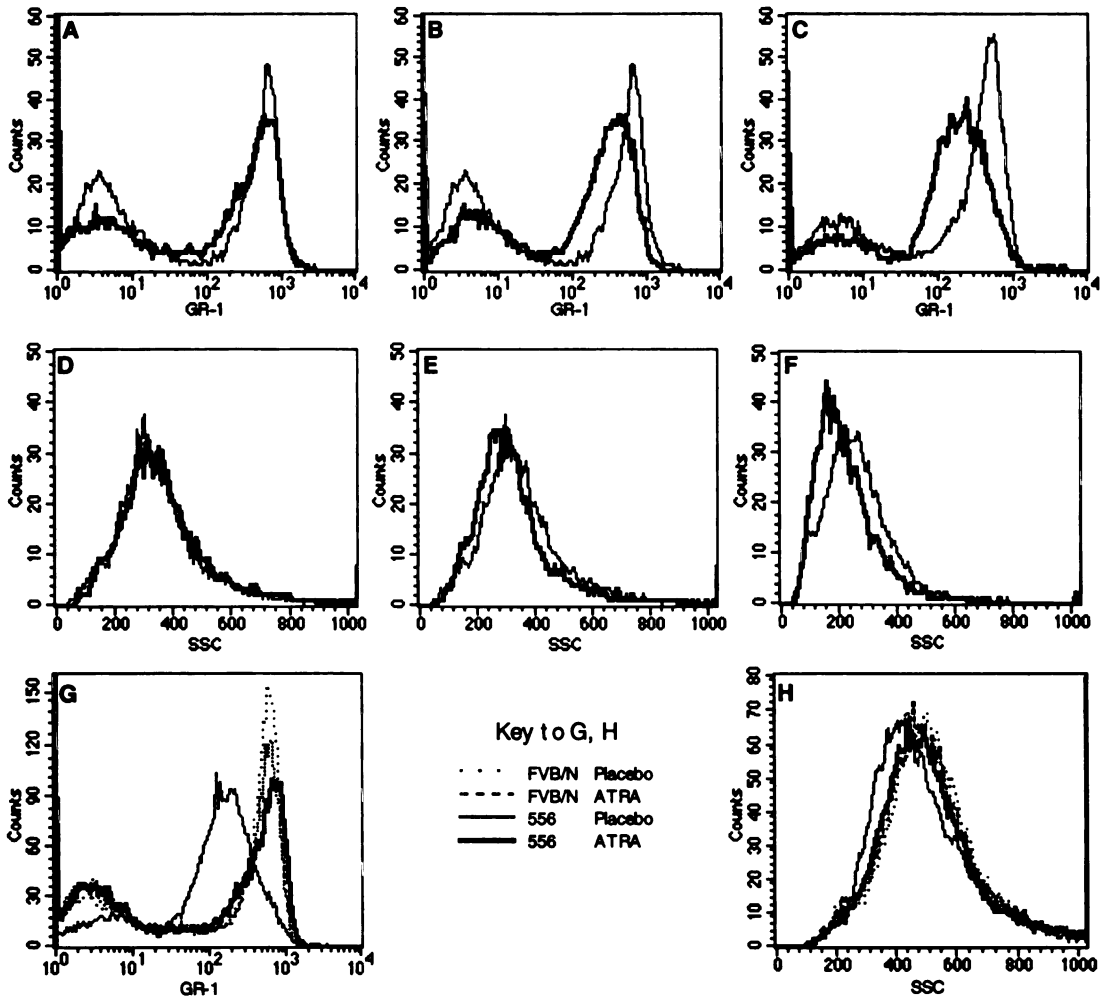
Figure 2.3 Abnormal granulocyte maturation in bone marrow of *PMLRARα* transgenic mice

Histograms from FACS analysis of bone marrow. (a-f) Samples from 2-4 month old *PMLRARα* transgenic mice (thick curves) were run in parallel with samples from age-matched control FVB/N mice (thin curves). 5 or more healthy mice from each line were examined with consistent results; representative samples are shown. (a-c) Bone marrow cells were stained with antibodies to Gr-1, a differentiation marker for murine myeloid cells. Gr-1 expression given as $\log_{10}$ fluorescence intensity. (d-f) Side-scatter profile of Gr-1 positive cells from samples a-c. (a,d) Line 553 expresses low levels of *PMLRARα*. (b,e) Line 565 expresses intermediate levels of *PMLRARα*. (c,f) Line 556 expresses high levels of *PMLRARα*. (g-h) Retinoic acid treatment shifts Gr-1 and side-scatter towards normal values. Placebo or 5 mg ATRA pellets were implanted into three week old FVB/N or line 556 mice; marrow was analyzed after 21 days. Each curve combines data from two mice. (g) Gr-1 (expression given as $\log_{10}$ fluorescence intensity). (h) Side-scatter of Gr-1 positive cells.

Figure 2.3

Abnormal granulocyte maturation in bone marrow of PMLRARα transgenic mice



neutrophil maturation but not others. For convenience, we refer to this condition as the "preleukemic state" throughout this report.

***PMLRARα* transgenic mice develop APL**

Twelve *PMLRARα* mice from five independent transgenic lines have developed APL when aged 3 to 8 months (median, 174 days) (Table 1). Assessment of lifetime penetrance of the leukemia has been precluded by the epidermal papillomatosis, which occurs in varying degrees in all the mice and makes it difficult to maintain mice for a normal life span (to be reported elsewhere). Nevertheless, it appears that penetrance of the leukemia may correlate with overall levels of transgene expression: while leukemias have been seen in lines with relatively low levels of *PMLRARα* expression, the disease has been more frequent in mice from the high-expressing 556 line (Table 1).

When the *PMLRARα* transgenic mice developed leukemia, they bled easily: routine intraperitoneal injection of anesthetic frequently led to a lethal hemorrhage. Schistocytes in blood smears provided further evidence of a bleeding diathesis. Peripheral blood counts were notable for anemia and thrombocytopenia in the absence of increased numbers of white blood cells (0.8-7.9 x 10^6 cells/ml in leukemias vs. 1.1-8.3 x 10^6 cells/ml in controls). A bleeding diathesis in the presence of normal or even low white counts is typical of human APL (Knowles 1992).

The pathology of the leukemia was consistent across the transgenic lines. Pale bone marrow was accompanied by lymphadenopathy and

TABLE 2.1

Relative levels of PmlRarα protein expression and hematopoietic phenotypes in transgenic mice

Line	Transgene expression		Hematopoietic phenotype			
Transgenic Founder	Western blot*	Immuno-fluorescence*	Abnormal FACS profile*	APL cases diagnosed in line	Age in days at diagnosis of APLs (median 174)	Mice in line reaching age 174 days‡
506	+	++	+	2	174, 195	45
553	-	+	+/-	3	78, 174, 265	79
556	++	+++	++	5	92, 143, 149, 176, 203	16
565	+	++	+	0	N/A	31
569	-	+	ND	1	155	12
608	-	+	ND	1	247	34

* Levels assigned based on visual comparison of blots, slides, or FACS histograms.

‡ Includes living mice 174 days or older as well as those aged at least 174 days at death (the median age at diagnosis of the twelve leukemias).

hepatosplenomegaly (Figure 2.4a). The enlarged lymph nodes had the greenish cast of granulocytic chloroma, characteristic of granulocytic infiltrates (King 1853; Knowles 1992). Microscopically, the mixed hematopoiesis of normal bone marrow was completely replaced by leukemic cells, splenic and lymphatic architecture was effaced, and leukemia extended throughout the hepatic parenchyma (Figure 2.4c). In some cases, extensive invasion of the kidneys, reproductive organs, or the meninges was also observed (data not shown).

Morphologically, the leukemic cells were promyelocytes (Figure 2.4e): they were large, had oval to indented nuclei and abundant basophilic cytoplasm with many primary granules. Mitotic figures and dysplastic nuclei were present. Although we have not observed any Auer rods or faggot cells in these leukemias, both of which are characteristic of human APL (Knowles 1992), we note that maturing human and murine granulocytes are morphologically distinct, and morphological variations might be expected between human and murine leukemias arising from this lineage. Like

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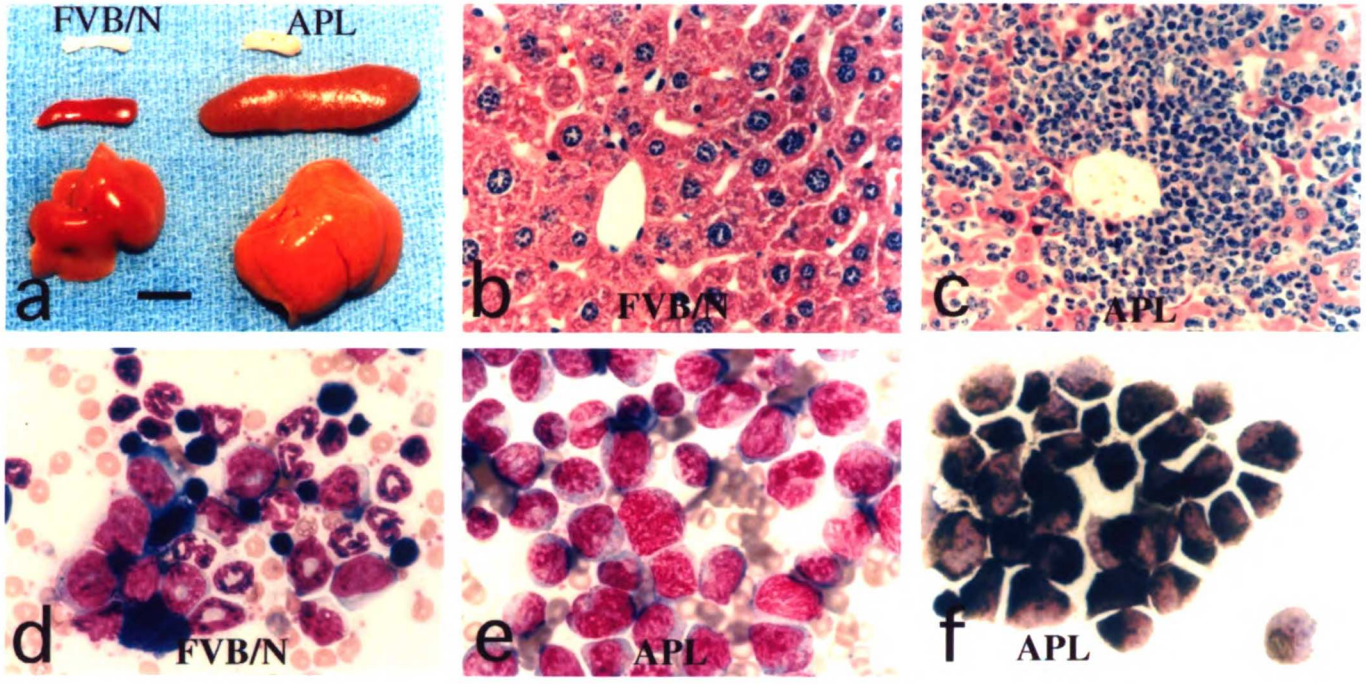
Figure 2.4 Pathology of APL

(a) Lymph node, spleen and liver taken from a control FVB/N mouse (left) are contrasted to those taken from a leukemia (right). Leukemic specimens were from first passage by transplantation of leukemia 802, line 556. Scale bar 1.0 cm. (b,c) Invasion of hepatic parenchyma (H&E, 100X original magnification). (b) Control FVB/N. (c) Leukemia (mouse 909, line 556). (d-f) Bone marrow isolates were stained with Wright's Giemsa and Azure B (d,e) or Sudan Black (f) (250X original magnification). (d) Control FVB/N. (e) Leukemia (mouse 1333, line 556). (f) Cytospun marrow cells from the second passage of leukemia 802 (line 556).

Figure 2.4
Pathology of APL



human promyelocytic leukemia cells, the murine leukemia cells stained strongly with Sudan Black B (Figure 2.4f), which marks the myeloperoxidase found in the primary granules of cells in the promyelocyte/granulocyte lineage (Davey 1989). The cells expressed low levels of Gr-1 and Mac-1 antigens (Figure 2.6b); low expression of CD11b, the surface marker detected by the Mac-1 antibody, is characteristic of APL cells (Paietta 1994). In 7-day methylcellulose culture, leukemic bone marrow gave rise to abundant compact colonies. Cytospins of cells isolated from these cultures principally contained promyelocytes (Figure 2.5a). We conclude that the transgenic mice had developed APL.

Murine APL and the preleukemic state are transplantable

To further verify the malignant character of the myeloid leukemia, we transplanted leukemic cells into unirradiated FVB/N mice. We intravenously injected cells isolated from the bone marrow or spleen of leukemic donors. To date, five leukemias have been tested with similar results: three to five weeks after transplantation, the recipient mice developed leukemia. Clinical and pathological features resembled the disease in the donors. Morphologically and by FACS analysis, the leukemic cells were identical to the donor cells. When analyzed by immunofluorescence, samples from the transplant recipients stained intensely with anti-Pml antibodies (data not shown). Since the recipient mice did not carry the transgene, this verified that the leukemic cells themselves expressed *PMLRARα*.

We were also able to transplant the preleukemic state by injecting bone marrow from young, healthy transgenic mice into lethally irradiated FVB/N mice. We have tested 6 of 47 recipient mice and all displayed marrow and blood FACS profiles characteristic of the transgenic line of the donor (data not

shown). We have seen 6 cases of APL in the recipients to date; 5 of these cases received marrow from the same donor, an F1 cross between two high-expressing transgenic lines (556x565). These results demonstrated that the observed hematological abnormalities were not a reaction to the papillomas, and provide the opportunity to define the incidence of leukemia and study tumor progression in a setting free of the epidermal disease.

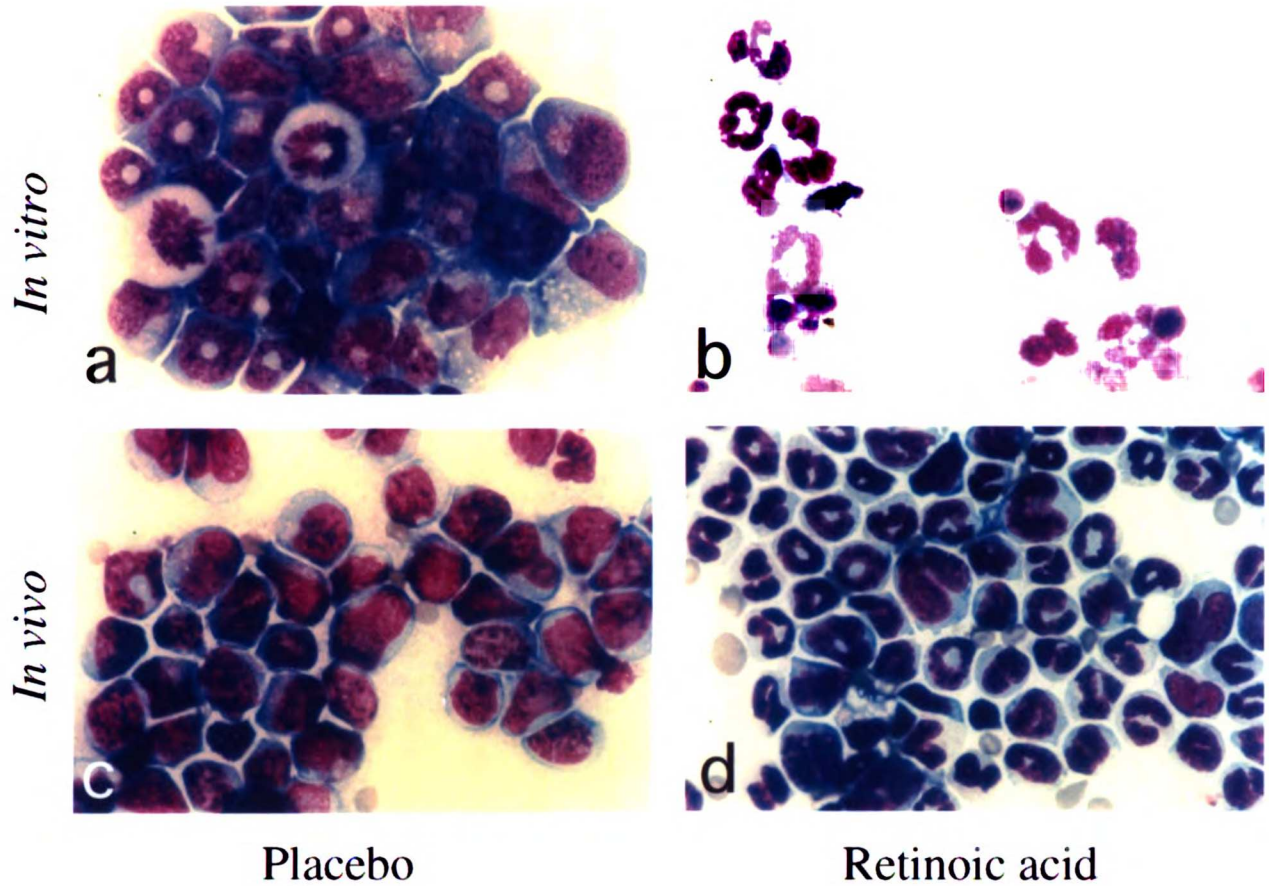
Murine APL differentiates in response to ATRA *in vitro* and *in vivo*

We wanted to determine whether the murine leukemia responded to ATRA like the human leukemia; the ability to passage the leukemias enabled us to generate additional material for these studies. When cultured for 7 days in the presence of ATRA, the compact colonies seen in untreated cultures were almost entirely replaced by colonies with an open, spreading appearance (data not shown). This suggested that ATRA was inducing differentiation, since mature neutrophils are mobile. Cytospins of treated cultures chiefly consisted of mature neutrophils (Figure 2.5b). These experiments demonstrated that the leukemic cells could be differentiated by ATRA treatment.

Figure 2.5 Leukemic cells differentiate into neutrophils in response to ATRA

Cytospins of fresh and cultured bone marrow cells were stained with Wright's Giemsa with Azure B. (a and b) 7-day Methylcellulose cultures from passage 1 of leukemia 877 (line 553) in the absence (a) or presence (b) of 1 μM ATRA. (c and d) Third passage of leukemia 877 (line 553). On day 11 after transplantation, 5 mg placebo (c) or ATRA (d) pellets were subcutaneously implanted. Samples were prepared on day 16 after transplantation. Original magnification 250X.

Figure 2.5
Retinoic acid differentiates murine APL



Next we looked at ATRA response in mice. We first used a suspension of ATRA in oil for intraperitoneal injection. The dose was calculated to approximate the blood levels achieved in human patients, with corrections for different route of administration and more rapid murine metabolism (Achkar 1994; Muindi 1994). ATRA and placebo injections were begun in 4 and 2 animals, respectively, on day 15 after transplantation, when promyelocytes were present in the peripheral circulation; on day 21 after transplantation, injections were stopped and one mouse killed due to onset of vitamin A toxicity.

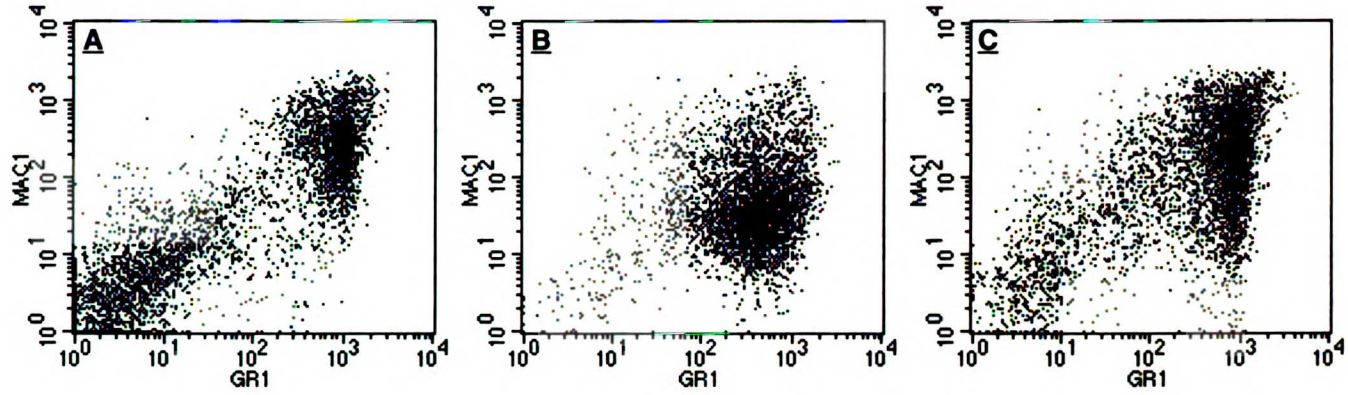
On day 28, a moribund placebo-treated mouse and a healthy ATRA-treated mouse were killed and their bone marrows were analyzed by FACS. Comparison of cells stained with Gr-1 and Mac-1 antibodies (Figure 2.6) showed two main clusters of cells in the FVB/N control marrow (Figure 2.6a): non-myeloid cells, negative for both markers, and maturing neutrophils, strongly positive for both markers. In the placebo-treated mouse (Figure 2.6b), the non-myeloid elements of the marrow were much reduced, and the bulk of the marrow was composed of leukemic cells expressing low levels of Gr-1

Figure 2.6 ATRA treatment of murine APL leads to remission

FACS analysis of Gr-1 and Mac-1 expression in bone marrow. Bone marrow cells from the control (a), placebo (b) and ATRA treated (c) mice were stained for Gr-1 and Mac-1 (murine CD11b) expression. Axes reflect $\log_{10}$ fluorescence intensity. (b,c) Samples from second passage by transplantation of leukemia 935 (line 569). On days 14-21 after transplantation, mice were given placebo (b) or 1.35 mg ATRA (c) by intraperitoneal injection of oil suspension. Cells were isolated on day 28 after transplantation. The control was an age-matched FVB/N.

Figure 2.6

ATRA treatment of murine APL leads to remission



and Mac-1. ATRA treatment (Figure 2.6c) restored a near-normal distribution of Gr-1 and Mac-1 staining.

The FACS changes were accompanied by the appearance of mature neutrophils in blood and bone marrow and by lessened hepatosplenomegaly and lymphadenopathy in treated mice. The two remaining ATRA treated mice were monitored to examine their long-term response to therapy. They continued in apparent remission until one relapsed 47 days after the end of treatment (68 days after transplantation), while the other remains in good health 7 months after transplantation.

Subsequently, we have subcutaneously implanted 21-day release pellets (containing either 5 mg ATRA or placebo) 10 to 20 days after transplantation. These obviated the need for daily injections and did not lead to overdose. In trials involving 6 to 12 animals each, we have tested recipients of transplants from 4 cases of leukemia, and have observed consistent responses to ATRA pellets. Within 3-5 days of initiating treatment, increasing numbers of neutrophils appeared in the peripheral blood and bone marrow (Figure 2.5d). The mice treated with the ATRA pellets generally did not survive much longer than those treated with placebos, but we suspect that their deaths were related to the pulse of differentiating neutrophils rather than to the leukemia itself, since maturing neutrophils were the predominant cell type in the bone marrows of treated mice. These adverse results of ATRA treatment may represent a murine version of the "retinoic acid syndrome", which is thought to be related to the rapid expansion of the neutrophil population induced by differentiation of the leukemic cells (Frankel 1992).

In addition to treating the leukemia with ATRA, we implanted ATRA or placebo pellets into transgenic mice that did not have leukemia. The distribution of Gr-1 expression and side-scatter was shifted towards normal

levels in 5/5 ATRA treated transgenic mice, while control FVB/N were unaffected by the treatment (Figure 2.3g-h). It thus appears that the drug overcomes the preneoplastic changes as well as the acute leukemia, reinforcing the hypothesis that ATRA can correct abnormalities elicited by expression of *PMLRARα* in neutrophils.

Discussion

PMLRARα impairs neutrophil differentiation

We have shown that expression of a human *PMLRARα* cDNA in murine granulocyte precursor cells alters their differentiation and initiates the development of APL. Some aspects of neutrophilic differentiation were sensitive to inhibition by PmlRarα, others were not. In particular, expression of the myeloid differentiation marker CD11b was not inhibited by PmlRarα, in accord with a previous report in which *PMLRARα* was expressed in U937 cells (Grignani 1993). Our *in vivo* results confirm the hypothesis that expression of *PMLRARα* in otherwise normal granulocytic cells impairs their maturation, and define a preleukemic state elicited by *PMLRARα*.

It could be argued that the severe epidermal papillomatosis seen in the transgenic mice might have secondary effects that could account for a disturbance in granulopoiesis. We do not believe that this is the case: we have seen consistent FACS abnormalities in transgenic mice as young as three weeks, in the absence of visible skin lesions, and preleukemic marrow transplanted into nontransgenic mice retains its characteristic phenotypic abnormalities and propensity to develop APL.

The molecular mechanisms by which PmlRarα inhibits granulocyte maturation are poorly defined. PmlRarα has been proposed to inhibit granulopoiesis by interfering with the normal activities of Pml or Rarα; alternatively, the juxtaposition of Pml and Rarα domains may lead to

anomalous transcriptional activation or other novel activities. While the function of Pml itself remains unclear, it has been characterized as a tumor suppressor in some systems (Mu 1994; Liu 1995) and PmlRarα might antagonize this activity. On the other hand, translocations involving *RARα* but not *PML* have been seen in a few cases of APL (Chen 1993b; Redner 1996), suggesting that alteration of Rarα function may be sufficient to initiate leukemogenesis. While some studies have supported the hypothesis that interference with Rarα function is sufficient (Tsai 1992; Tsai 1993; Onodera 1994; Onodera 1995), other results suggest that cooperation between the Pml and Rarα domains of PmlRarα yields a stronger phenotype (Grignani 1996). Our demonstration that PmlRarα impairs neutrophilic differentiation provides a means by which to clarify these issues: altered versions of the fusion gene can be expressed as transgenes under the *hMRP8* promoter and the resulting mice compared to the *hMRP8-PMLRARα* transgenic mice.

PMLRARα initiates APL

In addition to the preneoplastic changes in granulopoiesis, *hMRP8-PMLRARα* transgenic mice develop acute leukemia. We believe that expression of *PMLRARα* alone is not sufficient to cause leukemia because the leukemic phenotype combines incomplete penetrance with delayed onset. While the epidermal papillomatosis has limited median survival in the most severely afflicted transgenic lines to three to five months, we suspect the incidence of leukemia may be only a few percent over the normal life span of the mice (Table 1). Results with a small number of bone marrow transplants suggest a similarly low incidence. However, initial results suggest that crossing transgenic lines 556 and 565 yields higher expression of the transgene and a higher incidence of leukemia, without changing the latency. The three to nine month latency of the disease, taken together with the low penetrance

of leukemia, demonstrates that leukemogenesis by *PMLRARα* requires additional genetic changes.

Expression of *PMLRARα* may facilitate the acquisition of additional genetic changes by increasing the effective size of the myeloid compartment. Although the transgenic mice did not show a clear expansion of the compartment prior to the development of APL, increased proliferation might be balanced by increased destruction, the balance between differentiation and self-renewal might be altered, or genomic instability might be increased. These mechanisms could enhance accumulation of mutations that further block differentiation, promote proliferation, or decrease apoptosis of the promyelocytic cells. In collaboration with PmlRarα, these genetic changes could then release promyelocytes from normal growth constraints, resulting in the uncontrolled proliferation of malignancy.

The murine leukemia is an accurate model for human APL

We believe that the murine leukemia described here is a true analog of the human disease. APL in both species shares the promyelocytic character of the leukemic cells, a bleeding diathesis, and differentiation in response to ATRA (including both therapeutic and adverse effects). Our data further authenticate the role of *PMLRARα* in the specific pathogenesis of APL.

Other investigators have prepared mice in which transgenic *PMLRARα* is expressed by transcriptional controls for either the *CD11b* gene (Early 1996) or the human cathepsin G gene (*hCG*) (Grisolano). The *CD11b* mice failed to develop either preleukemia or leukemia. Their only apparent abnormality was an increased sensitivity to doses of irradiation that are normally sublethal, succumbing to infections because of protracted bone marrow suppression. Thus, it is not immediately apparent that these mice offer any access to the pathogenesis of APL.

In contrast, the *hCG* mice did display a preleukemic state that eventually progressed to overt leukemia, but they nevertheless differed in several substantive regards from the *hMRP8* mice described in the present communication. First, the preleukemia of the *hCG* mice featured intense accumulation of myeloid precursors that appeared otherwise normal, whereas the preleukemic changes in the *hMRP8* mice featured abnormal cells that did not accumulate in large numbers. Second, the clinical features of the leukemias in the two sets of mice differed appreciably. In particular, the *hMRP8* leukemias were accompanied by a bleeding diathesis, much as in human APL, and the leukemic cells showed no evidence of maturation. In contrast, no coagulopathy was noted in association with the *hCG* leukemias, and maturing myeloid cells continued to be evident in leukemic bone marrow and peripheral blood. Third, treatment with ATRA caused apoptosis of leukemic cells from the *hCG* mice, but differentiation of cells from the *hMRP8* mice, as if the underlying mechanism of pathogenesis might be fundamentally different in the two settings. Differentiation induced by ATRA led to remission of leukemia in the *hMRP8* mice. The effect of ATRA on leukemia in the *hCG* mice is not yet known.

Several factors may figure in these differences. Expression of the *PMLRARα* transgene is exceedingly weak in the *hCG* mice, in contrast to the relatively robust expression in the *hMRP8* mice. We have no credible explanation for the apparent paradox that the weaker expression gives rise to a more vigorous preleukemia. Second, it is likely that the transcriptional controls for the *hCG* and *hMRP8* genes target expression of the transgene to stages in myelopoiesis that are not entirely congruent. The *hMRP8* gene is expressed more broadly in the myeloid lineage than *hCG*, which is restricted to promyelocytes and promonocytes (Grisolano 1994). Thus, the cells in

which the leukemia initiates may differ in the two circumstances. Third, the *hCG* transgene may be down regulated following ATRA treatment, allowing the cells to apoptose, while expression from the *hMRP8* promoter is maintained during neutrophilic differentiation. Finally, the genetic backgrounds of the transgenic mice differ: the *hCG* mice were made in C57Bl/6 x C3H/He F2 cross mice, whereas the *hMRP8* mice were made in inbred FVB/N mice; there may be differences in the susceptibility to leukemogenesis by *PMLRARα* in the two strains.

In yet another experimental approach, an avian retrovirus was used to express *PMLRARα* in chick embryos (Altabef 1996). The result was an acute leukemia composed of multipotent blast cells, which were far less mature than the promyelocytes of APL (Altabef 1996). The blasts did not respond to ATRA, and their occurrence was associated with a consistent pair of mutations in the *PML* moiety of the fusion gene, mutations that have not been reported to occur in human patients. While this system did reveal that *PMLRARα* has leukemogenic potential outside a promyelocytic context, the recurrent mutations and non-promyelocytic character of the leukemic blasts distinguish it from human APL.

Future directions

The strong correspondence between human APL and the murine leukemia described here indicates that the *hMRP8-PMLRARα* system can be used to obtain information that is directly relevant to the human disease. The transgenic mice provide a valuable tool for further exploring the mechanisms of PmlRarα action, for isolating the additional genetic events that collaborate with *PMLRARα* to induce acute disease, and for exploring therapeutic approaches that may improve the survival of patients with APL and other leukemias.

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Chapter 3

Further investigations into the hematopoietic effects of *PMLRARα* transgene expression

Introduction

The analysis of the effects of *PMLRARα* expression, as discussed in the previous chapter, did not exhaust the list of questions that can be asked about *PMLRARα* and APL using the transgenic system. In this chapter, some additional data are presented and experiments are suggested to further our understanding of the preleukemic state, because much remains to be learned about how *PMLRARα* expression leads to leukemia. We would like to know which changes in neutrophilic differentiation are leukemogenic. Recent data from bone marrow transplant recipients make more precise calculations of the penetrance of the leukemic phenotype possible, suggesting that it may be higher than previously estimated. Crosses to other genetically altered mice are considered as they relate to the identification of events cooperating with *PMLRARα* in leukemogenesis. The advent of a new therapy for APL, treatment with arsenic compounds, offers an opportunity to examine the relationship between transgene expression and therapeutic response. Because the data presented are necessarily preliminary and incomplete, they are presented in a combined results and discussion format.

Results and Discussion

Which changes in neutrophilic differentiation are leukemogenic?

In order to know which changes in neutrophilic differentiation are leukemogenic, we need to better understand the effects of *PMLRARα*

expression on granulopoiesis. We have identified changes in surface marker expression and cytoplasmic granule characteristics that are due to *PMLRARα* expression. An interesting question is whether these reflect a global block in the neutrophilic differentiation program or a block in only subset of the program. Although antibodies to surface markers specific for neutrophilic differentiation are limited, we can look for changes in neutrophil function in *PMLRARα* transgenic mice.

There are many techniques that could be used to analyze the neutrophils' functional capacity (Chanarin 1989). Among these are assays that assess the ability of the neutrophil to produce bactericidal superoxides, such as nitroblue tetrazolium reduction or oxidative burst studies. Phagocytic activity of the neutrophils can be tested with opsonized latex beads. The ability of neutrophils to both phagocytose and kill bacteria can be analyzed with cultured *Staphylococcus aureus*. While these *in vitro* tests examine relatively discrete aspects of neutrophil function, the coordinated antibacterial effectiveness of the neutrophils can be tested by infectious challenge. The results of these functional studies should help to clarify which aspects of neutrophil maturation are affected by *PMLRARα*.

What is the leukemic stem cell in *hMRP8-PMLRARα* mice?

Native *hMRP8* expression patterns and experience with other *hMRP8* transgenic mice suggests that expression from *hMRP8* promoters may initiate earlier than the promyelocyte stage. As discussed in the previous chapter, comparisons between the *HCG* - and *hMRP8-PMLRARα* transgenic mice suggest that broader expression from the *hMRP8* promoter may be important: *PMLRARα* expression in precursors to promyelocytes may be leukemogenic.

We may be able to identify an earlier cell in which leukemia initiates by fractionating bone marrow from leukemic mice (which is abundantly

available from leukemia transplant recipients) and identifying the fractions that cause leukemia when transplanted to a new host. We can easily generate large numbers of leukemic donor mice by transplantation. The leukemic promyelocytes have a characteristic profile on FACS analysis with Gr-1 and Mac-1 antibodies, and the bulk of them could be eliminated by flow sorting. Markers for stem cells could be used to isolate very early precursors; cells expressing neither these very early markers nor the Gr-1 or Mac-1 antigens could also be isolated. The various fractions could then be injected into unirradiated FVB/N mice to determine which, if any, of the non-promyelocyte fractions was capable of transplanting the leukemia. These experiments could be complicated by the presence of contaminating promyelocytes in the other fractions, and careful quantitation would be necessary to interpret the results. If the experiment did identify an earlier cell that could transmit the leukemia, then the cells in that compartment could be more carefully analyzed in preleukemic marrow to determine what made them more susceptible to leukemogenic changes. For example, although we did not identify any substantial increase in promyelocytes in preleukemic transgenic bone marrow, there could be substantial increases in the population of a smaller, precursor compartment without our detecting it by standard marrow differential counts; such changes could be unmasked if we knew on which cells to focus.

The incidence of leukemia in recipients of *PMLRARα* bone marrow transplant recipients may approach 100%

Interim analysis of the recipients of bone marrow transplants suggests that the incidence of leukemia driven by *hMRP8-PMLRARα* may be higher than we previously thought. To date, we have transplanted bone marrow from six mice of transgenic line 556 into six lethally irradiated FVB/N recipients each.

Table 3.1 summarizes causes of death in the recipient mice and the age of the marrow at death, calculated from the date of birth of the donor mouse. Of the 36 recipient mice, 2 died of early graft failure, 4 died of APL with a latency of 4 to 9 months, and 20 died of other causes, particularly radiation induced tumors; 10 remain in good health. It has been nearly a year since the marrow donors were born, and the leukemia free survival, measured as (living mice)/(living mice + leukemic mice), is presently 71%.

Table 3.1

Fate of recipients of transplanted line 556 bone marrow

Bone marrow donor	Causes of death as of 12/22/96	Days since birth of marrow donor
1097	Died of APL	202
1097	Died of non-APL related problem	235
1097	Died of non-APL related problem	255
1097	Alive	321
1097	Alive	321
1097	Alive	321
1098	Early death/graft failure	62
1098	Died of non-APL related problem	100
1098	Died of non-APL related problem	100
1098	Died of non-APL related problem	196
1098	Died of non-APL related problem	235
1098	Died of APL	290
293T	Early death/graft failure	98
293T	Died of APL	126
293T	Died of unknown causes	152
293T	Died of unknown causes	152
293T	Died of APL	274
293T	Alive	383
297T	Died of non-APL related problem	238
297T	Died of non-APL related problem	238
297T	Died of non-APL related problem	266
297T	Alive	383
297T	Alive	383
297T	Alive	383
462T	Died of non-APL related problem	199
462T	Died of non-APL related problem	199
462T	Died of non-APL related problem	208
462T	Died of non-APL related problem	234
462T	Died of non-APL related problem	295
462T	Died of non-APL related problem	301
467T	Died of non-APL related problem	232
467T	Died of non-APL related problem	313
467T	Died of non-APL related problem	325
467T	Alive	353
467T	Alive	353
467T	Alive	353

Summary

Cases of APL	4
Survivors (at least 10 months after birth of donor)	10
Deaths not due to APL	22
Transplants performed	36

Although we have not checked all of the mice that survived the irradiation and transplantations to verify that they reconstituted from donor marrow, all those that we did examine did carry donor marrow. Inclusion of mice that did not reconstitute with donor marrow, however, would lead to our underestimating the incidence of leukemia. If the leukemias did not arise independently in each donor, but rather were transplanted from a leukemic donor to several recipients, we would overestimate the incidence of leukemia in the recipient cohort. Because the leukemias arose after several months' latency and were not clustered among recipients of leukemia from a single donor, however, they probably represent cases arising independently in the recipient mice.

We have also performed bone marrow transplants from a few mice resulting from a cross between lines 556 and 565. These mice were bred to increase levels of the transgene, and probably represent the maximum viable level of *PMLRARα* expression (homozygotes of line 556 have not been seen in 556 x 556 crosses and are presumed inviable). We could not maintain 556x565 transheterozygotes for long due to their extreme epidermal phenotype and therefore have studied their marrow in transplants. In our first experiment, bone marrow from two transheterozygotes was pooled for transplant into 6 irradiated recipients. One recipient died of early graft failure, and the other five all developed APL with a latency of 5 to 7 months (dated from the birth of the marrow donors). This represents a 100% incidence among the mice that survived the transplant procedure. No mice were successfully reconstituted in a second trial of 565x556 marrow. These transplantation results, if replicated, imply that the development of APL may be inevitable if *PMLRARα* expression is high enough.

Despite the very high level of *PMLRARα* expression in these mice, there was still a substantial latency in the onset of the leukemias, arguing that additional mutations are still required before overt disease occurs. Higher levels of transgene expression may further increase the likelihood of accumulating these additional mutations.

Candidate gene approaches to identifying genes cooperating with *PMLRARα* in leukemogenesis

In addition to using reverse genetics to identify genes that cooperate with *PMLRARα* in the development of APL, the mice can be directly crossed with other mice that carry transgenic versions or have deficiencies of likely genes. We have initiated crosses to mice bearing activated *NRAS* or *BCL2* under the control of the *hMRP8* promoter, and we plan crosses to mice deficient in retinoblastoma-gene family members Rb, p107, and p130.

NRAS* probably does not significantly accelerate leukemogenesis by *PMLRARα

Activation of *RAS* genes are a common event in human myeloid leukemias (Bos 1989). Although no studies have found a specific link between *RAS* activation and APL, we decided to ask whether activated *RAS* would accelerate development of APL. Mice expressing an activated *NRAS* mutant (G12D) under the *hMRP8* promoter have developed lymphoid lesions, subcutaneous neuroendocrine tumors, and skin lesions similar to those seen in the *PMLRARα* mice; while *NRAS* is expressed in myeloid cells of these mice, no myeloid leukemias have been documented (Kogan, S., personal communication). When line 7 *hMRP8-NRAS* transgenic mice were bred to line 556 *hMRP8-PMLRARα* mice, the offspring that bore both transgenes developed an extreme epidermal papillomatosis, much worse

than that seen with either transgene alone. The bone marrow of these mice was therefore studied in recipients of bone marrow transplants.

Marrow from five donors was transplanted into six recipients each. Three recipients did not reconstitute their marrow, five developed APL, and eleven mice were euthanized for causes other than development of APL. Approximately one year after the marrow donors were born, the leukemia-free survival (calculated as before) was 63% in this cohort. The incidence of leukemia in these mice is not appreciably different than in the *PMLRARα* mice, suggesting that *NRAS* does not significantly cooperate with *PMLRARα*. It is still possible, however, that even though both transgenes involved in the cross were under control of the same promoter, they were not expressed together in the cell in which APL initiates, or that higher expression levels of the *NRAS* transgene in those cells would have led to a different outcome.

BCL2 may cooperate with _PMLRARα_ to accelerate leukemogenesis

We have crossed the *hMRP8-PMLRARα* mice with *hMRP8-BCL2* mice to examine the effects of decreased apoptosis on leukemogenesis by *PMLRARα*. *PMLRARα* expression alone can inhibit apoptosis in some cells (Grignani 1993; Rogaia 1995), and *BCL2* inhibited apoptosis of neutrophils in *hMRP8-BCL2* mice (Lagasse 1994). If there was a strong increase in leukemogenesis, it would suggest that *BCL2* and *PMLRARα* have additive anti-apoptotic effects, and that apoptosis of leukemic precursor cells limits the rate of progression to leukemia.

We have not yet generated a substantial number of double transgenics carrying *hMRP8-BCL2* and *hMRP8-PMLRARα*. In one litter, two out of three *BCL-2/PMLRARα* pups appeared to have a worse skin phenotype than the *PMLRARα* mice, while the other did not. We decided to perform bone marrow transplants to avoid complications arising from the skin lesions.

Marrow from two doubly transgenic donors was transplanted to six irradiated transplant recipients each. Approximately five months after the donor mice were born, and almost four months after transplantation, eight of the twelve mice are still alive; three have developed APL, and one healthy recipient was sacrificed for a detailed examination of its bone marrow. The healthy mouse, sacrificed three months after transplantation, carried a substantial increase in promyelocytes in the bone marrow; very few morphologically mature neutrophils were seen in the peripheral blood.

These results suggest, but do not yet confirm or rule out cooperativity between *BCL2* and *PMLRARα*. At present, the cross appears to result in accumulation of higher numbers of promyelocytes in healthy mice while the number of leukemias is similar to that seen in recipients of marrow transplants from *PMLRARα* mice. If these results were confirmed after following a larger cohort for a longer period of time, it would indicate that further accumulation of promyelocytes is not a rate limiting step in leukemogenesis by *PMLRARα*.

Retinoblastoma family members are good candidates for cooperation with *PMLRARα*

Loss of the retinoblastoma protein is a common event in many tumors (Weinberg 1995). A recent report has suggested that loss of retinoblastoma protein function frequently occurs in acute promyelocytic leukemia (Paggi 1995). Interestingly, several proteins known to bind Rb or Rb-related p107, p130, or p300 have been documented to localize to Pml-containing nuclear bodies (Carvalho 1995; Szekely 1996), and the LXCXH motif, which plays an important role in binding to the Rb family members, is required for this localization (Szekely 1996). One of the Rb-binding proteins, Ebna5, can sometimes be localized with Rb itself in a nuclear speckled pattern

reminiscent of Pml NBs (Jiang 1991). If a cross between *hMRP8-PMLRARα* mice and mice bearing a deficiency for *RB* or another *RB*-family member yielded accelerated leukemogenesis, the links between Pml-associated nuclear bodies and Rb family members may reveal the accelerating mechanism.

Issues in APL therapy: murine APL cells apoptose with arsenic treatment

The advent of a new therapy for APL, As_2O_3 (Chen 1996), which appears to cause apoptosis rather than differentiation of the leukemic cells, has widened the therapeutic options for APL patients. We have initiated studies of As_2O_3 therapy in our murine APLs, to determine whether they respond like human APLs. In vitro treatment of murine APL cells with 1μM arsenic does lead to apoptosis, further underlining the similarity between the murine model and the human disease. However, our initial treatments *in vivo* , with 4 to 6 recipients of transplanted APL in each, have not led to any increased survival of the leukemic animals. We daily injected 5 μg As_2O_3 subcutaneously from day 12 after transplantation in the first trial and 10 μg from day 12 after transplantation in the second trial. In both cases, illness in the treated animals followed the same clinical course as the untreated animals. When the moribund animals were killed, their marrows were filled with promyelocytes whether or not they had received As_2O_3. Higher doses, a different route of administration or more frequent dosing may be required to replicate the *in vitro* response to As_2O_3*in vivo*.

Is PmlRarα degradation a necessary prerequisite for a therapeutic response to APL treatments?

Studies on As_2O_3 administration *in vivo* have shown that As_2O_3 leads to increased degradation of the PmlRarα protein (Chen 1996), which has been suggested to be the cause of the apoptotic effect of the treatment. Several

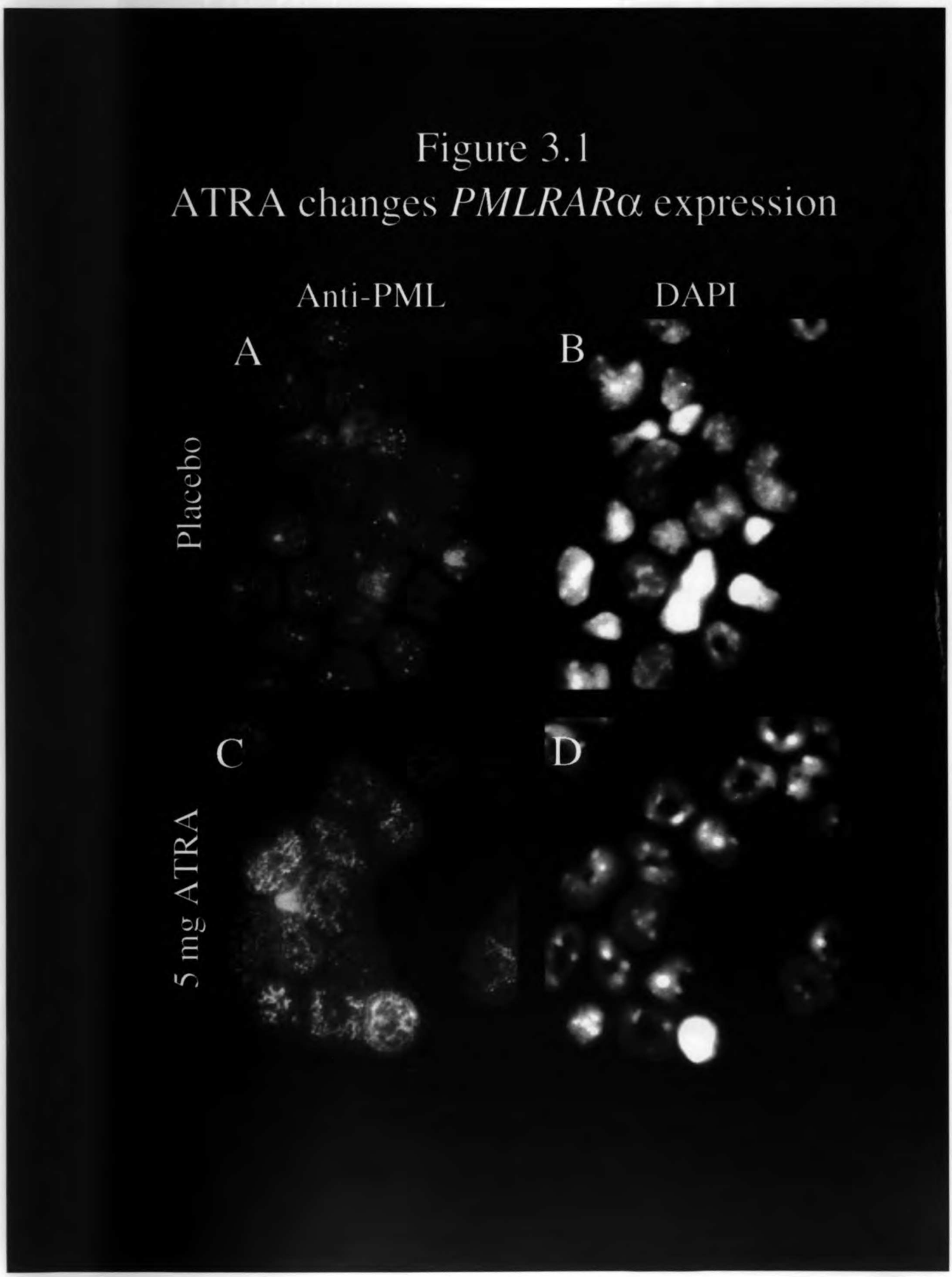
groups have recently reported decreased levels of PmlRarα in promyelocytes after ATRA treatment, which does not trigger apoptosis (Raelson 1996; Yoshida 1996). Yet another report, however, suggests that interferon treatment, which increases *PMLRARα* expression, enhances the responsiveness of NB4 cells to ATRA (Nason-Burchenal 1996). It therefore remains unclear whether PmlRarα degradation should be a goal of anti-APL therapies.

While we have not yet systematically determined whether PmlRarα is degraded during ATRA treatment, we should be able to address this issue using the murine APLs. We have used anti-PML antisera in immunofluourescent staining of leukemic cells from animals treated with placebo or retinoic acid (Figure 3.1). We saw what appeared to be an increase in the fluorescent signal in cells from ATRA treated animals: there were more bright nuclear speckles in the cells from the ATRA treated animals than from the placebo treated animals. An increase in *PMLRARα* expression would be consistent with the effect of differentiation on the *hMRP8* promoter, but the brightness of immunofluorescence is not necessarily directly proportional to the amount of antigen present. Western blotting of bone marrow samples from ATRA and placebo treated animals should yield a more definitive answer as to whether differentiation is occurring in the presence of decreasing, stable or increasing PmlRarα levels.

Figure 3.1 Pml expression appears to increase after ATRA treatment.

Bone marrow cells from recipients of transplanted leukemia were stained with anti-PML antiserum (a,c) and DAPI (b,d). (a,b) Leukemia 877, passage 4, untreated. (c,d) Leukemia 877, passage 3, after ATRA treatment. Original magnification 250X.

Figure 3.1
ATRA changes *PMLRARα* expression



Defining effective ATRA regimens for therapy of murine APL

In the previous chapter, we discussed two different ATRA treatment regimens for murine APL: daily intraperitoneal injections of drug in an oil vehicle, and subcutaneous implantation of timed-release pellets. The only trial which yielded long term survivors (one of whom has now survived for 7 months since treatment) used intraperitoneal injection.

The timed-release pellets did succeed in differentiating the leukemic cells, but the ATRA-treated animals rarely outlived the placebo-treated animals for more than a few days. At necropsy, their marrows were typically packed with differentiating neutrophils instead of promyelocytes, but they were nevertheless ill. We have attributed this phenomenon to the retinoic acid syndrome, which may be related to the very high neutrophil counts that follow ATRA treatment (Frankel 1992). But we have not documented any specific pathologic findings associated with the syndrome. We can look for signs of the syndrome, such as pulmonary edema, at necropsy and in pathological specimens from the ATRA-treated leukemic mice.

Further studies of the murine APL would be aided by a standardized treatment that can reliably lead to remission. The timed-release pellets should be an adequate and convenient form of treatment if timed and dosed appropriately. Among the parameters that should be considered is the stage of the disease at the initiation of treatment, because animals with very high peripheral blood counts are likely to develop a retinoic acid syndrome. The dose and duration of drug delivery by the pellets is probably also important: the 5 mg /21 day timed-release product may not be delivering enough drug fast enough. In the trial of injected drug that was successful, 1.35 mg was delivered to each mouse daily for seven days. Over the week, then, each mouse received 10.5 mg of ATRA (treatment was stopped after 7 days due to

signs of drug toxicity). A 5 mg/21 day pellet would be expected to release only 1.7 mg/7 days, which may not be enough to arrest the progress of the disease. A first trial giving the animals pellets releasing 10 mg/21 days showed promise, as the treated animals lived longer than the controls. It may be possible to increase the dose to 15 or 20 mg/ 21 days for improved response without substantial toxicity.

Chapter 4

Expression of *PMLRARα* in the skin of transgenic mice leads to papillomas

Introduction

Retinoids have long been recognized to be important to maintenance of normal skin (Frazier 1931; Jarrett 1980; Rook 1992). In their absence, skin becomes hyperkeratotic and follicles are disrupted (Frazier 1931; Wechsler 1979; Rook 1992). An excess of retinoids also leads to a hyperkeratotic phenotype, but one characterized by increased proliferation throughout the epidermis (Jarrett 1980; Rook 1992).

As discussed in the previous chapters, transgenic mice were generated expressing the *PMLRARα* fusion gene under the control of the *hMRP8* promoter. The *hMRP8-PMLRARα* mice developed hyperkeratotic, papillomatous skin lesions in addition to, and apparently independent of, their hematopoietic phenotype. These lesions appear to represent the results of expression of the *PMLRARα* transgene in the epidermis of the mice.

Expression of the transgene in the epidermis may be a natural consequence of the *hMRP8* promoter specificity. In addition to expression in the myeloid lineage (Dale 1983; Hayward 1986; Dorin 1987; Odink 1987), the Mrp8 protein has been identified in a number of stratified squamous epithelial tissues (Gabrielsen 1986; Wilkinson 1988). Although several studies failed to demonstrate the presence of Mrp8 in the bulk of normal

epidermis, expression was seen in pilosebaceous structures (Figure 4.0) (Gabrielsen 1986; Brandtzaeg 1988). Expression has also been documented in keratinocytes in diseased skin, particularly when accompanied by inflammation, and in squamous cell carcinomas of the skin (Gabrielsen 1986; Wilkinson 1988).

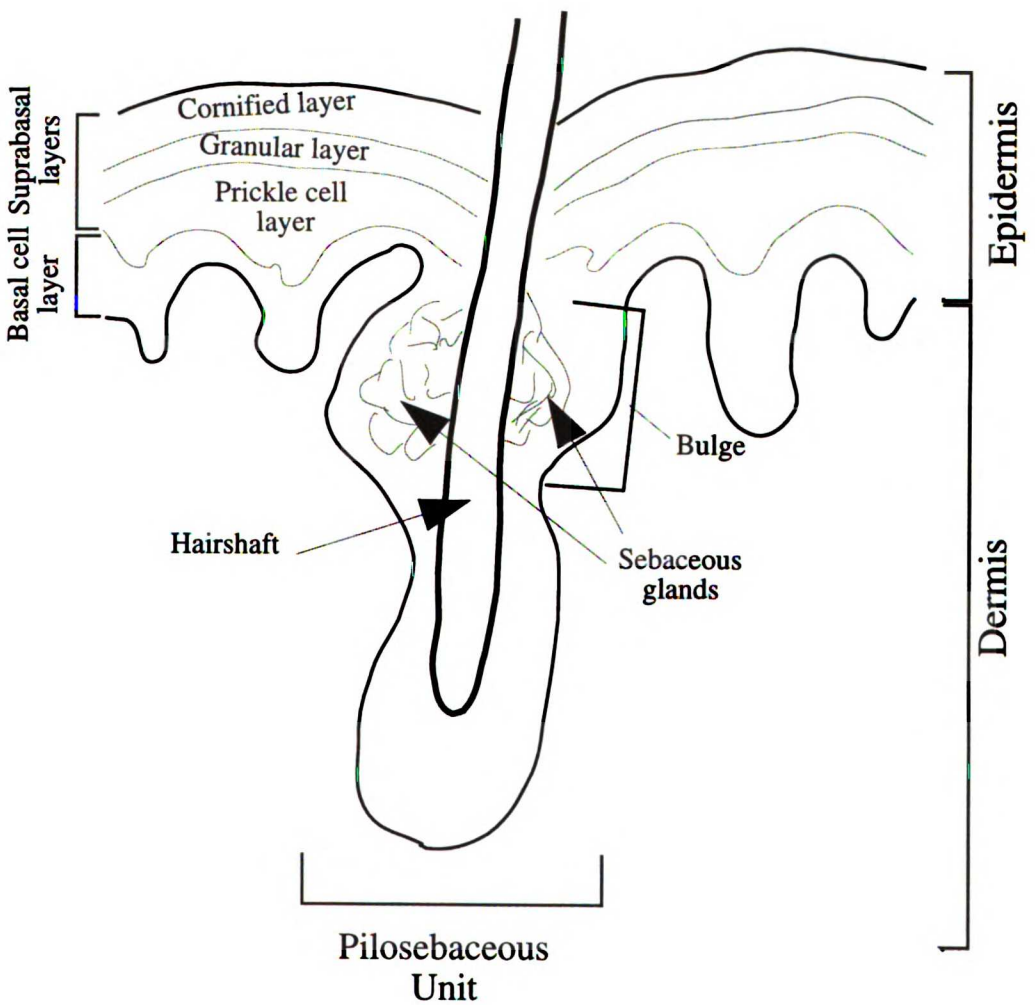
The spectrum of abnormalities seen in the skin of the *hMRP8-PMLRARα* transgenic mice ranged from minimal thickening of the skin surrounding the ear tag to large, verrucous lesions up to a centimeter in diameter. These papillomatous lesions strongly resembled human keratoacanthomas, a typically benign, self-limiting hyperplasia of epidermis (Pinkus 1981; Rook 1992). In at least one case, a papilloma progressed to malignancy. Retinoic acid treatment prevented the development of epidermal lesions, suggesting that PmlRarα may be interfering with a pathway common to epidermal and myeloid differentiation.

Figure 4.0 Organization of the epidermis and hair follicles

The epidermis consists of the proliferative basal cell layer and the non-proliferative suprabasal layer and cornified cell layer. The suprabasal layer is often further subdivided into the prickle (or spinous or stellate) cell layer and the granular cell layer, which appears as a dark line beneath the cornified layer on H&E stained sections. The pilosebaceous unit consists of basal cells surrounding a hairshaft and sebaceous glands. The thickened region of the follicle near the sebaceous glands, known as the bulge, contains epidermal stem cells.

Figure 4.0

Organization of the epidermis and hair follicles



Results

The *hMRP8-PMLRARα* transgenic mice develop skin lesions

In ten of eleven founder transgenics or their offspring, we observed a consistent syndrome of epidermal hyperplasia. The abnormalities varied from slight thickening at the site of the ear tag to regions of thickened, hairless, corrugated skin to many distinct papillomas covering a large portion of the animal's body (Figure 4.1). 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 lesions by age 6 weeks, and their lesions were generally more severe than those seen in the low-expressing lines. Mice bred to have increased transgene

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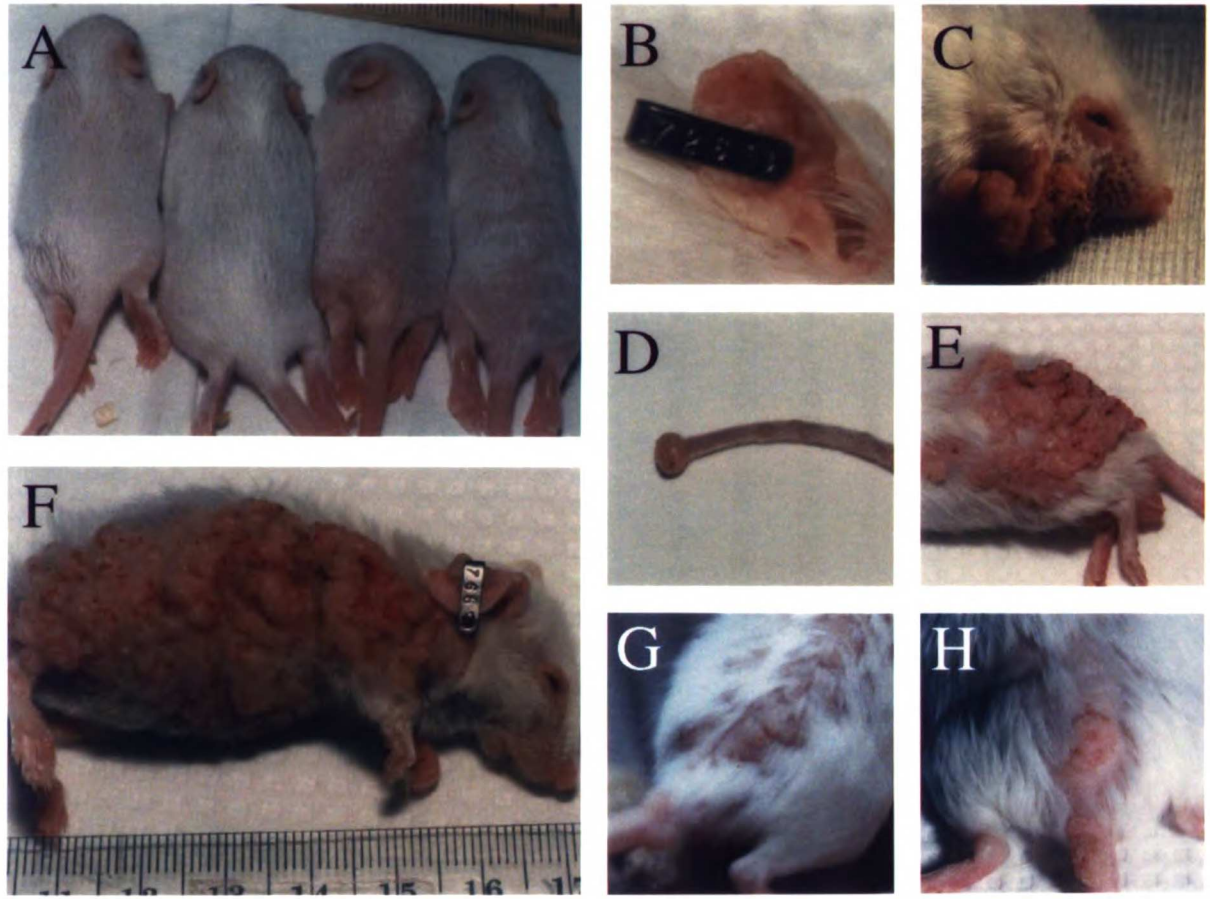
Figure 4.1 Spectrum of epidermal lesions in *hMRP8-MLRARα* mice

(a) These 11 day old pups were sired by a hemizygous line 556 mouse. At this age, the transgenic pups (pair on right) can easily be distinguished from their non-transgenic littermates (pair on left) by their thinner, more irregular hair coat. In older mice, distinct lesions are typically found at the ear tag site, ranging from a minor hyperplasia (b) to a large tumor (c). Tumors often develop at the site of tail biopsy (d), bite wounds (e), and in one extreme case extended over most of the torso of the animal (f). In the absence of discrete tumors, hyperkeratotic regions often take on a corrugated appearance (g); this type of involvement sometimes resolves into hairless scar with a few discrete tumors (h). (b) Mouse 726, line 553, age 5 months. (c) Transgenic founder 556, age four months. (d) Mouse 705, line 556, age 3.5 months. (e) Mouse 727, line 553, age 4 months. (f) Mouse 765, line 556, age 2 months. (g and h) Mouse 506, line 506, age 6 weeks (g) and 8 months (h).

Figure 4.1
Common skin lesions in *PMLRARα* transgenics



dosage generally had more severe phenotypes: in some lines, crosses of two heterozygotes resulted in roughly 1:2:1 distribution of unaffected, equally affected, and more severely affected offspring (Figure 4.2). In cases where the more severely affected animals were viable to breeding age (e.g., lines 553 and 565), lines of mice have been established that appear to be homozygous for the transgene. These results strongly support the hypothesis that the skin lesions were due to transgene expression.

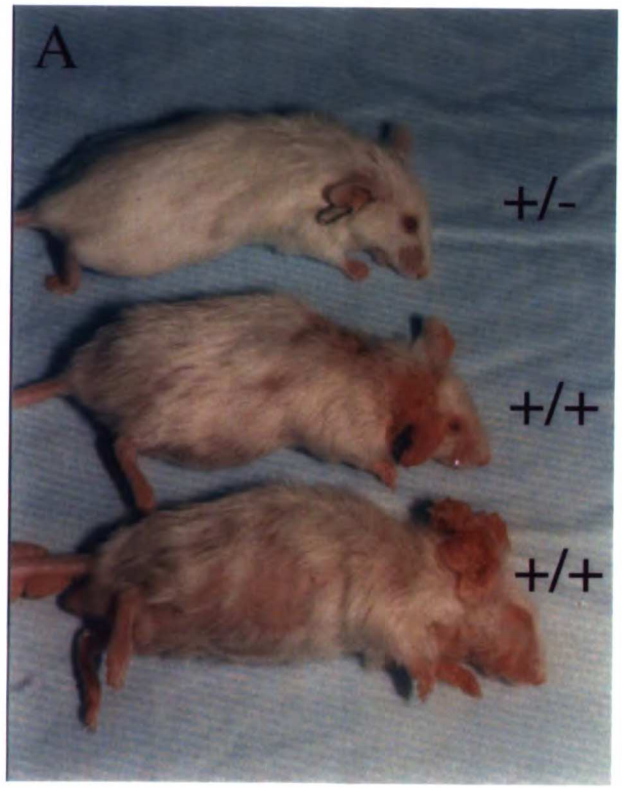
Because these lesions were only seen in the transgenic individuals, and not in recipients of transgenic bone marrow transplants, we believe that these lesions were not secondary to the hematopoietic phenotype but rather represented the direct effect of the *hMRP8-PMLRARα* transgene expression in the skin. Mice bearing activated *NRAS* under the *hMRP8* promoter have also developed epidermal lesions consistent with *hMRP8*-driven transgene expression in the skin (Kogan, S., personal communication).

Although we have not explicitly confirmed *PMLRARα* expression in the skin to date, preliminary results from a pilot study using immunohistochemistry suggested that the transgene may be expressed in basal keratinocytes and pilosebaceous structures (data not shown).

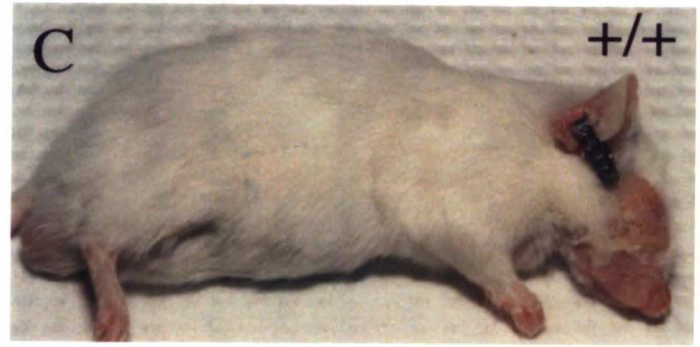
Figure 4.2 Epidermal phenotype displays dose-response relationship with transgene expression

Mice of line 553 (a) and line 565 (b,c) have two distinct patterns of epidermal phenotype. Based on the segregation of these patterns in crosses within each line, they appear to correspond to hemizyosity (a, top, and b) and homozygosity (a, middle and bottom, and c) for the transgene. (a) From top: mice 1868, 1938 and 1941, aged 5 months. (b) Mouse 265T, age 2 months. (c) Mouse 1254, age 2 months.

Figure 4.2
Dose response to transgene expression



Line 553



Line 565

Additional studies to verify transgene expression in the epidermis are in progress, including further immunohistochemistry with anti-Pml antibodies, and may include western blotting or RT-PCR as needed.

Transgenic skin is abnormal prior to the development of papillomas

In additional to the papillomas, other differences can easily be distinguished between the skin of *hMRP8-PMLRARα* transgenic and non-transgenic mice. For example, in the line with the highest transgene expression, 556, litters of pups can be accurately typed at 11 days: the transgenic pups have a sparser haircoat which clearly differentiates them from their non-transgenic littermates (Figure 4.1a). Histologically, the epidermis of the transgenic mice was characterized by regions of hyperaplasia: in 11 day old pups, fewer hair follicles appear 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 (Figure 4.3b).

In older mice, cells accumulated in the hair follicles, particularly in the bulge region midway between the follicle base and the skin surface, where

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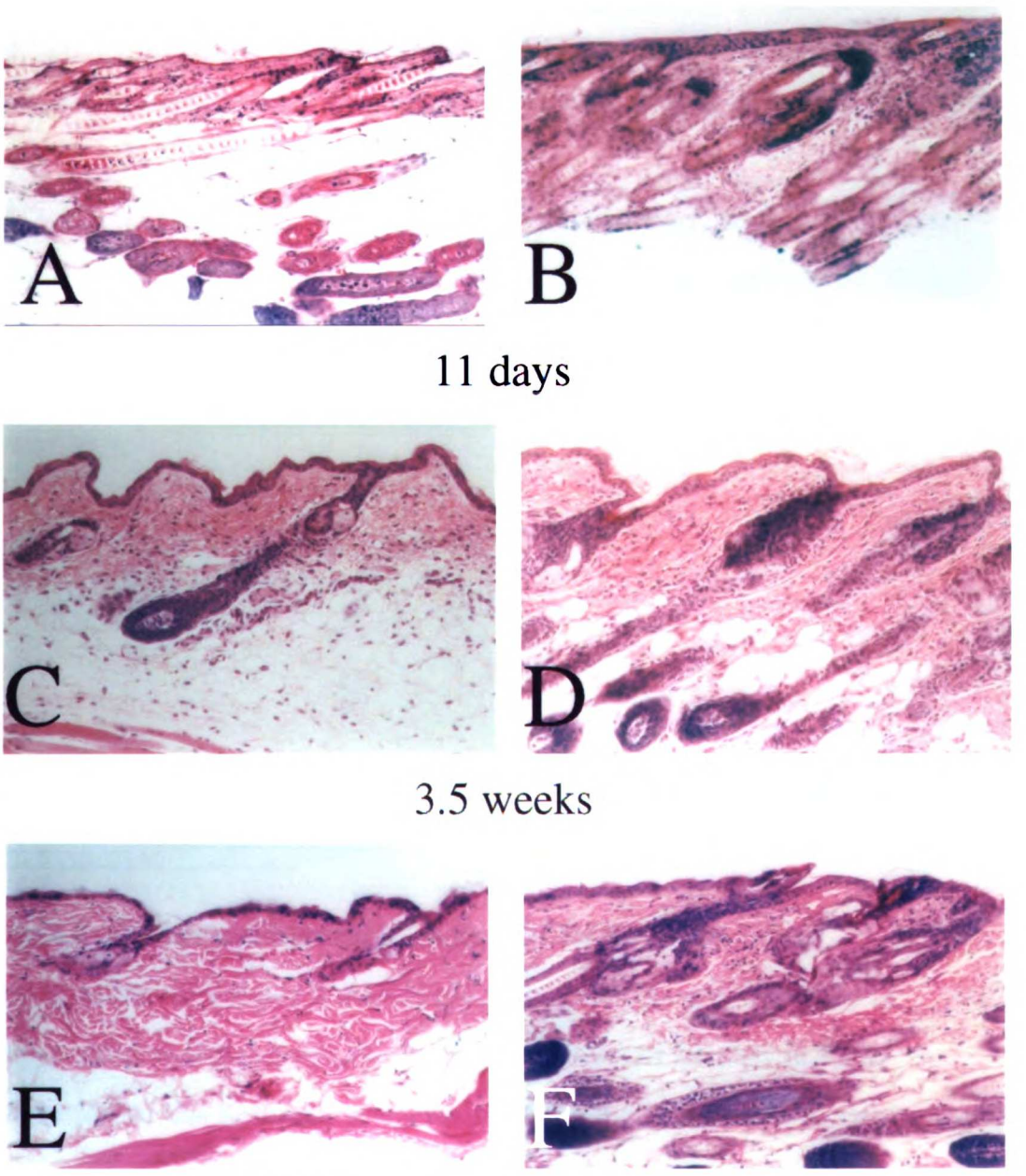
Figure 4.3 Histology underlying visually normal skin in line 556 transgenic mice

Differences between line 556 transgenic mice (b,d,f) and nontransgenic FVB/N (a,c,e) are apparent throughout life, even in regions of grossly normal skin. Fewer hair follicles breach the surface of 11 day old skin (b). In adult mice, more cells are present in the bulge region of the hair follicle (d), and irregular, hyperplastic sebaceous glands are common (f). The mice were ages 11 days (a,b), 3.5 weeks (c,d) and 3 months (e,f). H & E, original magnification 80X.

Figure 4.3
Histology underlying visually normal skin
in line 556 transgenic mice



follicular stem cells may reside (Figure 4.3d, f) (Cotsarelis 1990). Sebaceous and keratinizing differentiation could be seen amid the accumulated bulge cells. Follicles could be found that appeared to represent continuum from normal to precursors of papilloma (Figure 4.4): first, accumulation of cells in the bulge; second, excessive keratinizing in the follicle ducts; and third, disruption of the follicle with a keratinaceous plug. The interfollicular epidermis displayed patchy thickening with occasional apoptotic or dysplastic cells.

We have begun to characterize the proliferative state of the transgenic epidermis by looking at BrdU incorporation and PCNA expression. These techniques may uncover an increased proliferative tendency in the transgenic epidermis. Preliminary results with BrdU incorporation pointed to higher

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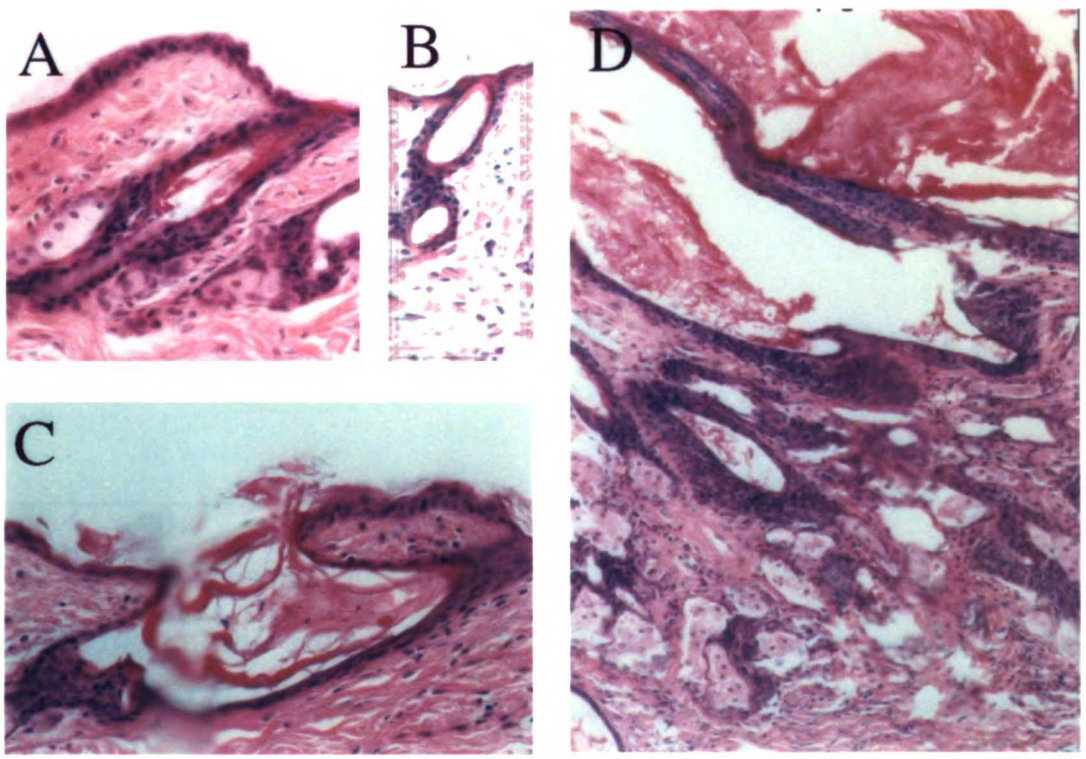
Figure 4.4 Abnormal hair follicles in *PMLRARα* mice

The epidermis of these 3 month old mice displays a continuum of follicular abnormalities from minor accumulation of keratin (a,b), to degeneration of the hairshaft and expansion of the keratinaceous center (c) to disorganized masses of keratin (d), which prefigure the keratinaceous cysts present in larger lesions. (a-d) Mouse 1889, line 556, age 3 months. H&E, original magnification 160X.

Figure 4.4
Abnormal follicles in *PMLRARα* mice



proliferative index in the bulge cells in transgenic skin (Figure 4.5b), which could account for the increased size of the bulge. Increased proliferation of the basal cells was also seen in the papillomatous lesions (Figure 4.5d,e).

PMLRARα transgenic mice develop papillomas

Histologically, the large lesions that developed in the *PMLRARα* transgenic mice were papillomas, with hyperplastic papillary fronds that

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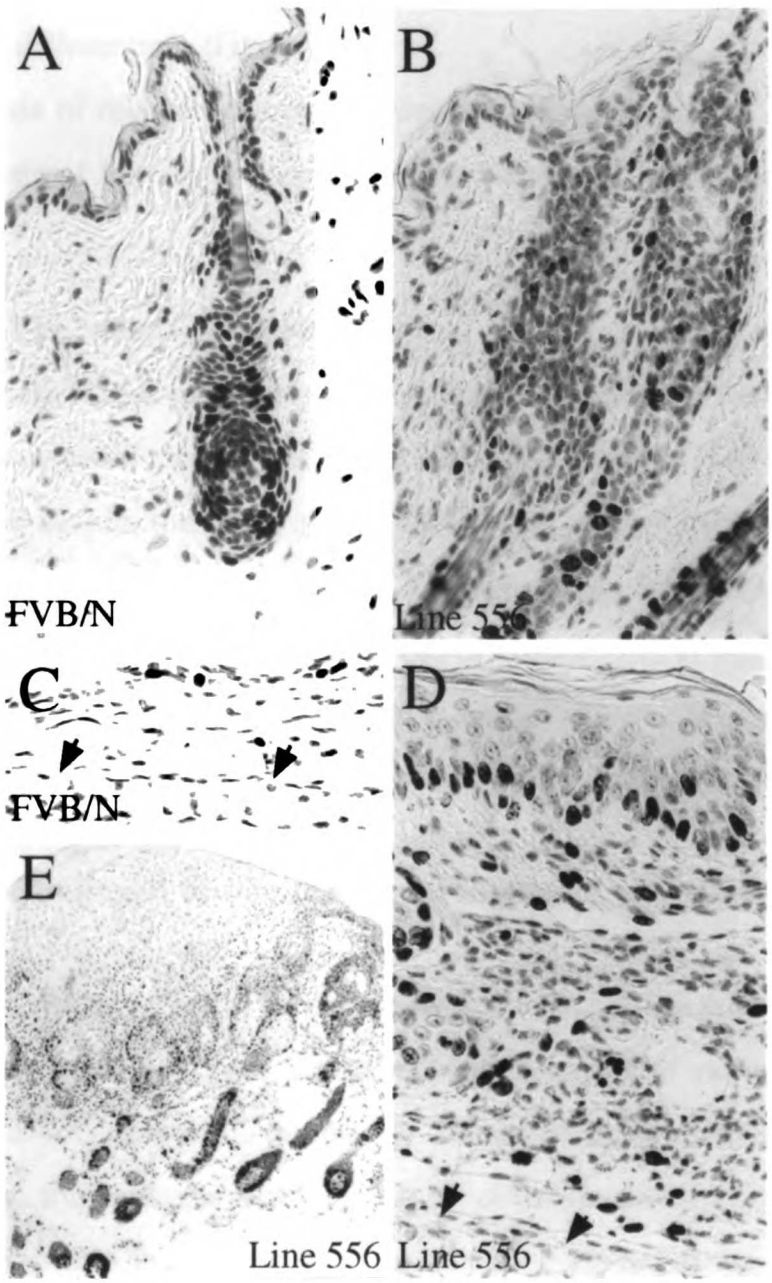
Figure 4.5 Proliferation in *PMLRARα* epidermis

Antibodies to BrdU label more cells in the epidermis of transgenic mouse 769 (b,d,e) than non-transgenic mouse 775 (a,c). There may be more labelled cells in the vicinity of the bulge region of the hair follicles in the transgenic skin (b). Close to the ear tag, many more cells are labelled in the hyperplastic transgenic skin (d) than the non-transgenic skin (c). Note the much greater thickness of the transgenic skin from the surface to the cartilage (arrows). Original magnification 160X. (e) In this lesion from mouse 769, the basal cells surrounding keratinaceous cysts are labelled strongly but the upper strata are not. Nuclei were counterstained with hematoxylin (blue). Original magnification 40X.

Figure 4.5
Proliferation in *PMLRARα* epidermis



accumulated thick coats of keratin (Figure 4.6). Keratinaceous cysts and plugged hair follicles were major features of most lesions. Inflammation was frequently but not inevitably present. There was generally hyperplasia throughout the epidermis, in which the different strata were thicker but continued to differentiate (Figure 4.7).

Hyperplasia of relatively normal appearing cells throughout the epidermis is characteristic of human epidermal lesions known as keratoacanthomas.

Figure 4.6 The lesions are papillomas

Despite their varied gross appearance, the papillomatous lesions in the skin of the transgenic mice are histologically very similiar, with continued differentiation despite the hyperplasia in all layers of the epidermis. H & E. (a) Lesion from the back of mouse 1889, line 556, at age 3 months. Original magnification 20X. (b) Lesion from the ear of mouse 1166 at age 3 months. Arrows indicate the ear cartilage. Original magnification 10X. (c) Lesion from the ear of mouse 556 at four months. Original magnification 20X.

Figure 4.7 Continued differentiation throughout papillomatous epidermis

(a, b, d) Views of a typical ear lesion from a 3 month old transgenic mouse, at increasing magnification are contrasted with a region of ear epidermis from a 1 month old non-transgenic mouse (c). At approximately the same magnification, the cartilage and the stratum corneum of the epidermis are encompassed within the picture of the normal ear (c), but at the same magnification, the photo of the lesion in the transgenic mouse (d) barely encompasses the epidermis. H&E. (a, b, d) Mouse 1095, line 556. (c) FVB/N. Original magnification 10X (a), 40X(b), and 100X(c,d).

Figure 4.6
Representative large lesions

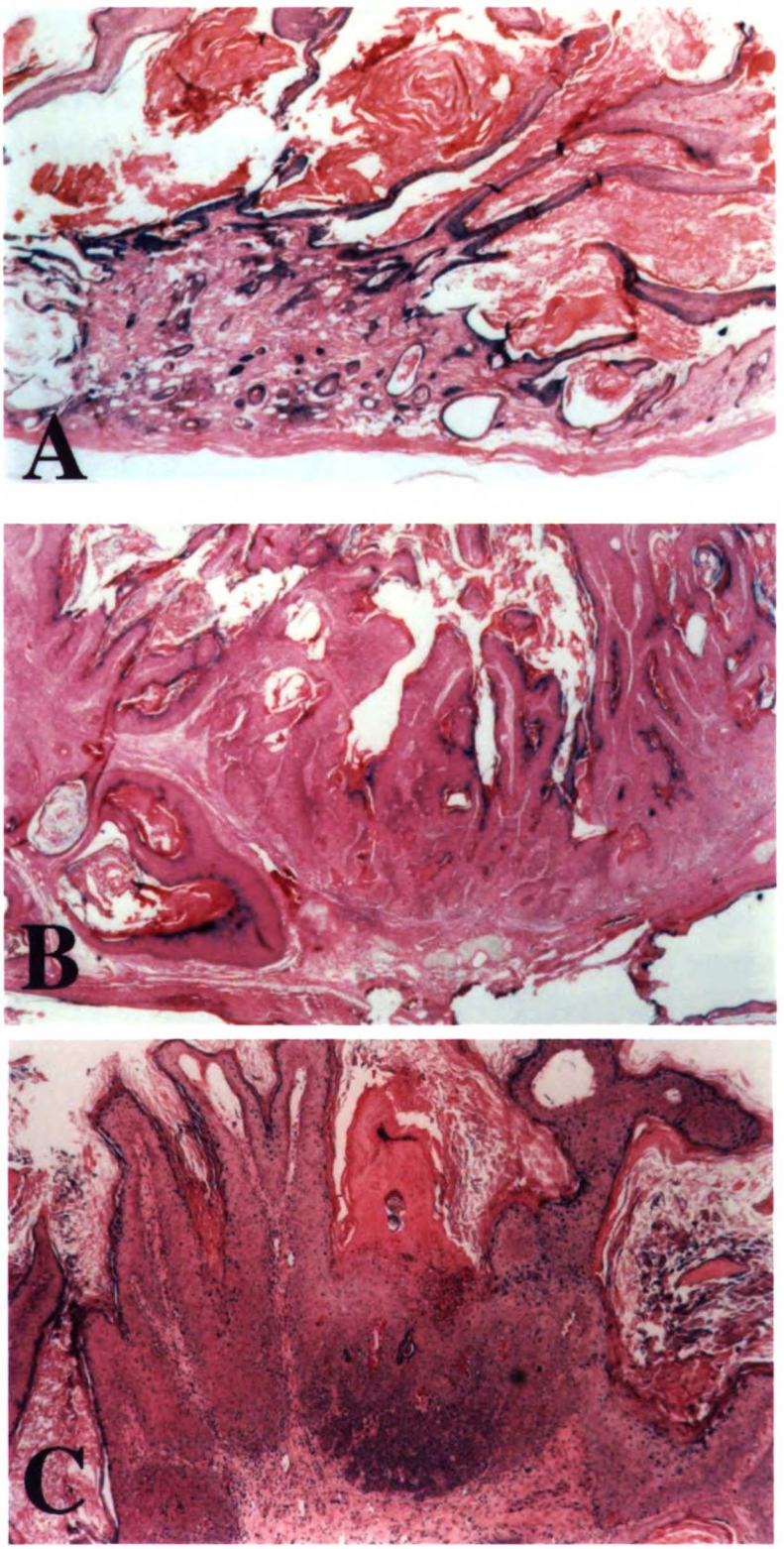
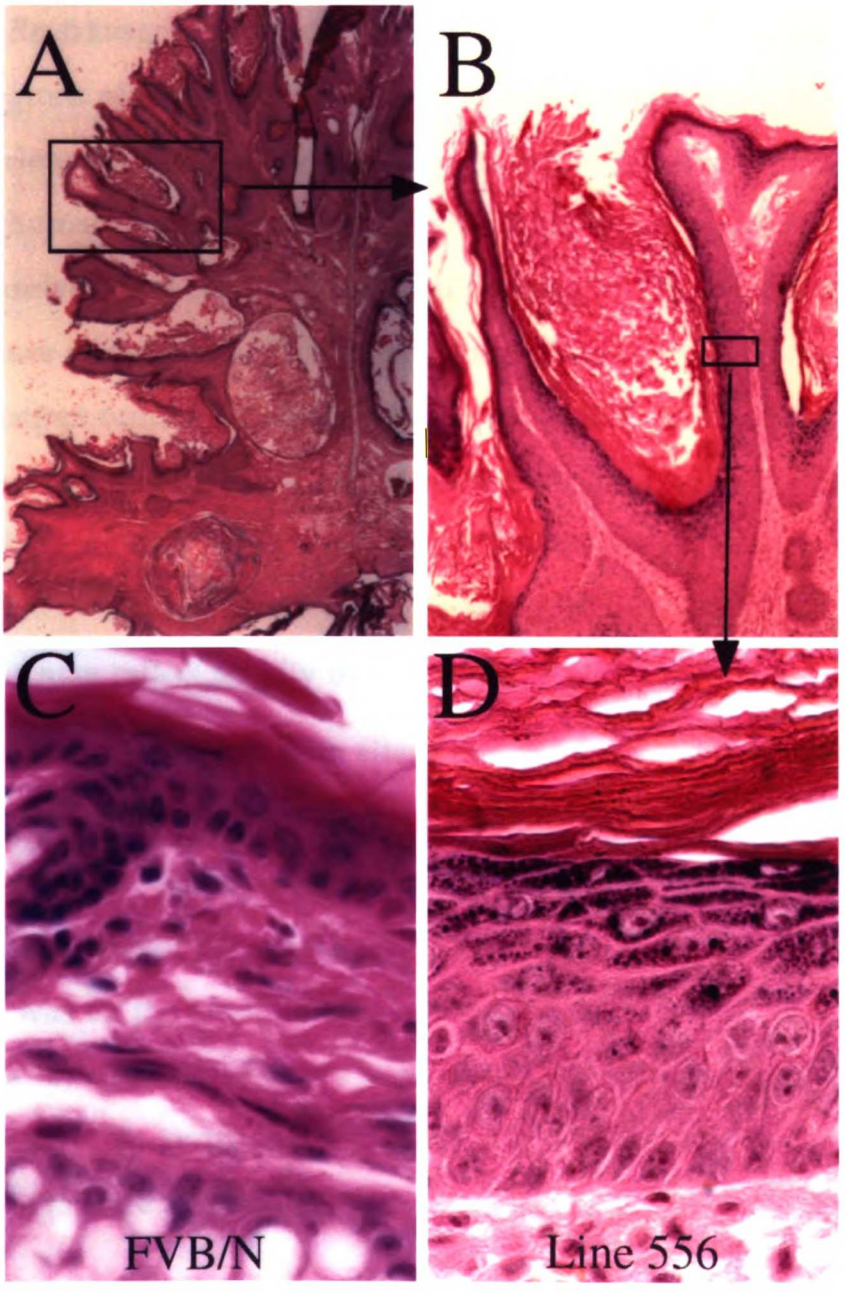


Figure 4.7

Differentiation throughout papillomatous epidermis



Keratoacanthomas in humans are considered benign, self-limiting lesions: although they closely resemble squamous cell carcinomas grossly, histologically, and molecularly, their clinical course is distinctly different. Keratoacanthomas grow more rapidly but typically resolve, without treatment, into quiescent, fibrotic patches of scar tissue (Pinkus 1981; Rook 1992; Kerschmann 1994; Vahlquist 1996). Consistent with this clinical picture, *PMLRARα* transgenic mice developed visible tumors as early as 3 weeks of age. Regions of thickly furrowed hyperkeratotic skin frequently resolved into thin hairless patches (Figure 4.1g,h), consistent with the resolution typically seen in human keratoacanthoma. Keratoacanthomas do not always resolve by themselves, however, and metastatic lesions with the histologic appearance of keratoacanthomas have been documented (Hodak 1993). In the mice, distinct tumors frequently remain in areas where the bulk of the lesions have resolved (Figure 4.1h), but no metastases have been documented from any of the skin lesions in the MPR8-*PMLRARα* mice. Some lesions incorporated very high levels of Brdu; others incorporated less (Figure 4.5 d,e): this may reflect activity or quiescence in the tumors.

Papillomas are associated with sites of wounding or inflammation

The papillomas were not randomly distributed over the bodies of the transgenic mice. The most common site, at which the largest tumors were seen, was the site of the ear tag (Figure 4.1b,c). The next most common location was on the hindquarters, where mice are apt to bite each other (Figure 4.1e). Tumorous knobs at the site of tail biopsy were also common (Figure 4.1d). To verify that wounding could trigger tumor formation, wound clips were attached to the back skin of six-week-old FVB/N and

transgenic mice (Figure 4.8a). After 12 days, the clips were removed. Substantial lesions were already present in the transgenic mice (Figure 4.8d). The slight inflammation present in the control mice (Figure 4.8a) resolved after the clips were removed (Figure 4.8a,b), but the tumors persisted and continued to grow in the transgenic animals (Figure 4.8 c,d). These results indicated that wounding could stimulate the growth of papillomas in the transgenic mice.

The papillomas can progress to malignancy

One particularly large papilloma contained areas of profound hyperplasia of the basal layer of the epidermis (Figure 4.9a). In some regions, there appeared to be invasion of the dermis by basal cells with dysplastic features

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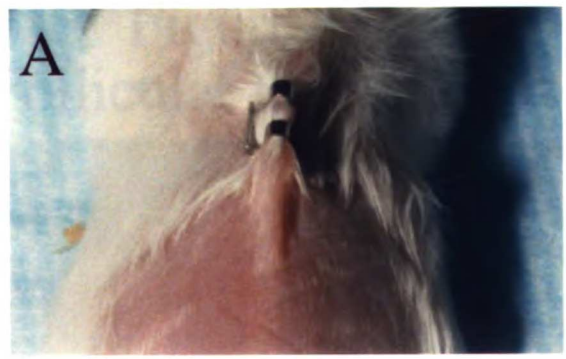
Figure 4.8 Wound response

A wound clip was attached to back skin of six to eight 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). (a,b,c) FVB/N mouse 312T. (d,e) Mouse 301T, line 556.

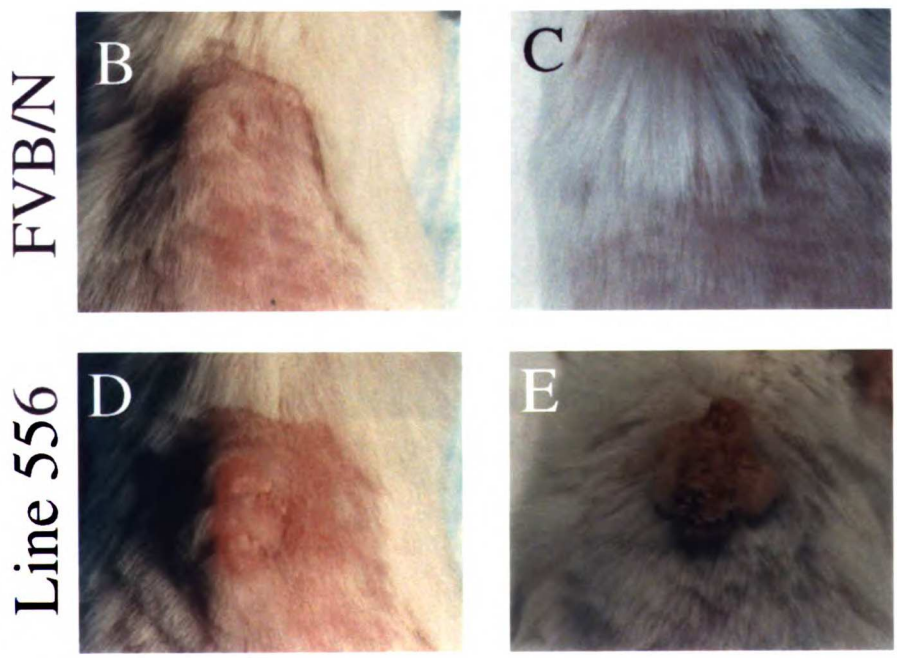
Figure 4.9 Follicular carcinoma

In this very large tumor from the ear of mouse 556, nests of basal cells have invaded the dermis (a). The box shows the approximate location of the area magnified in (b), in which dysplastic basal cells occasionally show sebacous differentiation (arrow). The combination of dysplastic basal and sebaceous cells marks this as a follicular carcinoma. H&E. (a) Original magnification 20X. (b) Original magnification 160X.

Figure 4.8
Wound response



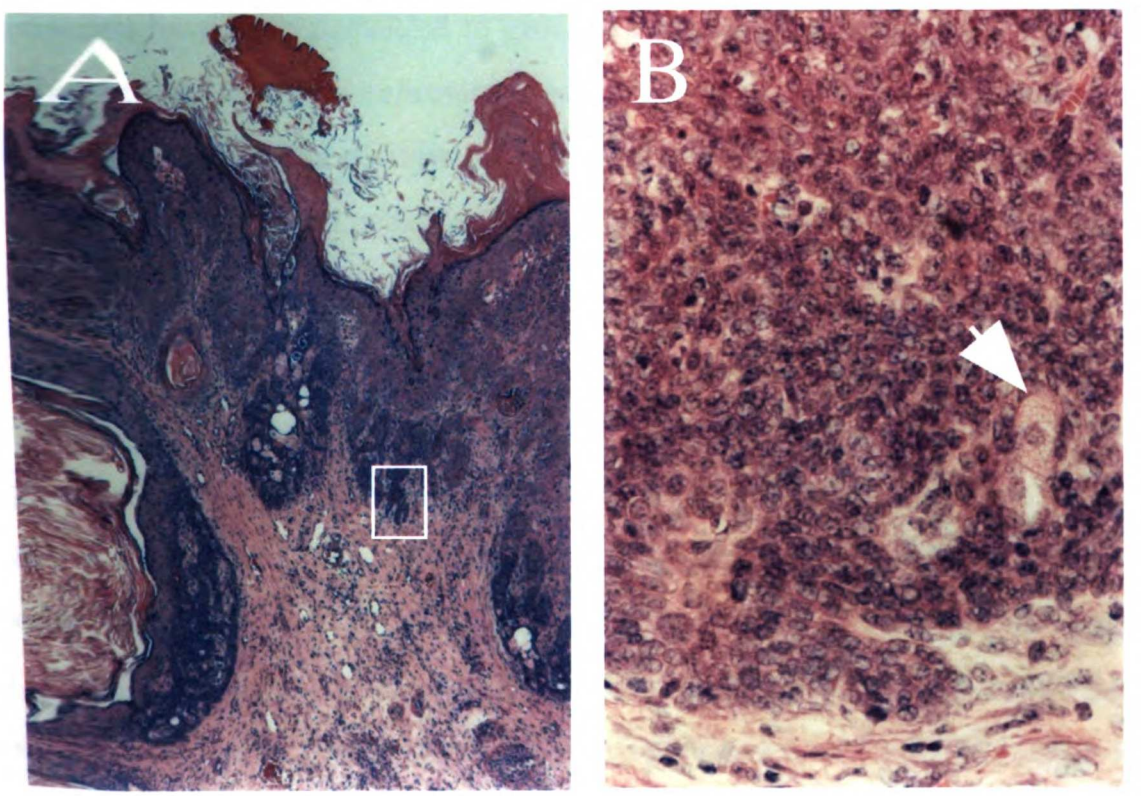
Day 0



12 days

6 weeks

Figure 4.9
Follicular Carcinoma



(Figure 4.9b). These features demonstrated that this lesion contained areas of malignancy. Because occasional cells within this region showed sebaceous differentiation, it might represent a follicular carcinoma. At present, we have not examined enough tumors to determine whether the malignant progression seen in this tumor was an isolated case. This tumor, excised from mouse 556 at approximately three months of age, was unusually large, and was excised because it was interfering with the animal's activities. Few other tumors have been permitted to grow to a similar size, which could limit the opportunity to observe metastasis even if it was otherwise a common event. However, regions of disrupted basal membrane, occasional dysplasia in basal cells, and intermixed sebaceous cells have occurred in several other tumors. These regions might be close precursors to follicular carcinomas.

Retinoic acid is effective prophylaxis against papilloma formation

We were interested to discover whether retinoic acid could normalize epidermal differentiation as it did the altered hematopoiesis. These studies were undertaken with subcutaneous implantation of time-release pellets containing ATRA. The initial experiment was inconclusive: pellets releasing 0, 0.05, or 5 mg of ATRA over three weeks were each implanted in two 4-6 week old mice of transgenic line 556. There was no consistent reduction in existing lesions or clear difference in the generation of new tumors at the implantation site in the transgenic mice, and no tumors were formed in the control FVB/Ns. There was, however, an exuberant inflammatory response with encapsulation of the pellets, seen chiefly in the transgenic mice, which might have impaired absorption of the drug.

In order to avoid the confounding inflammatory response seen in the 556 mice, placebo or 5 mg ATRA pellets were each implanted in 3 15-day old pups of line 565 which were homozygous for the transgene. Heterozygotes of this

line rarely developed papillomas outside of the ear tag site, while 100% of homozygotes developed hyperkeratotic lesions around their eyes and muzzles within 6-8 weeks after birth (Figure 4.2c). Three weeks after the pellets were implanted, the animals that received the placebo developed lesions in the characteristic pattern, but the others had not (Figure 4.10). Retinoic acid therefore was able to inhibit the development of the lesions.

NRAS accelerates development of skin lesions in *PMLRARα* mice

As discussed in the previous chapter, *MRP8-PMLRARα* transgenic mice were crossed with *MRP8-NRAS* mice to determine whether the *PMLRARα* and activated *NRAS* would cooperate in leukemogenesis. While the progeny of the cross did not appear to have a stronger hematopoietic phenotype than the *PMLRARα* mice, the skin phenotype was substantially worsened. *PMLRARα/NRAS* mice could not be maintained beyond five weeks of age

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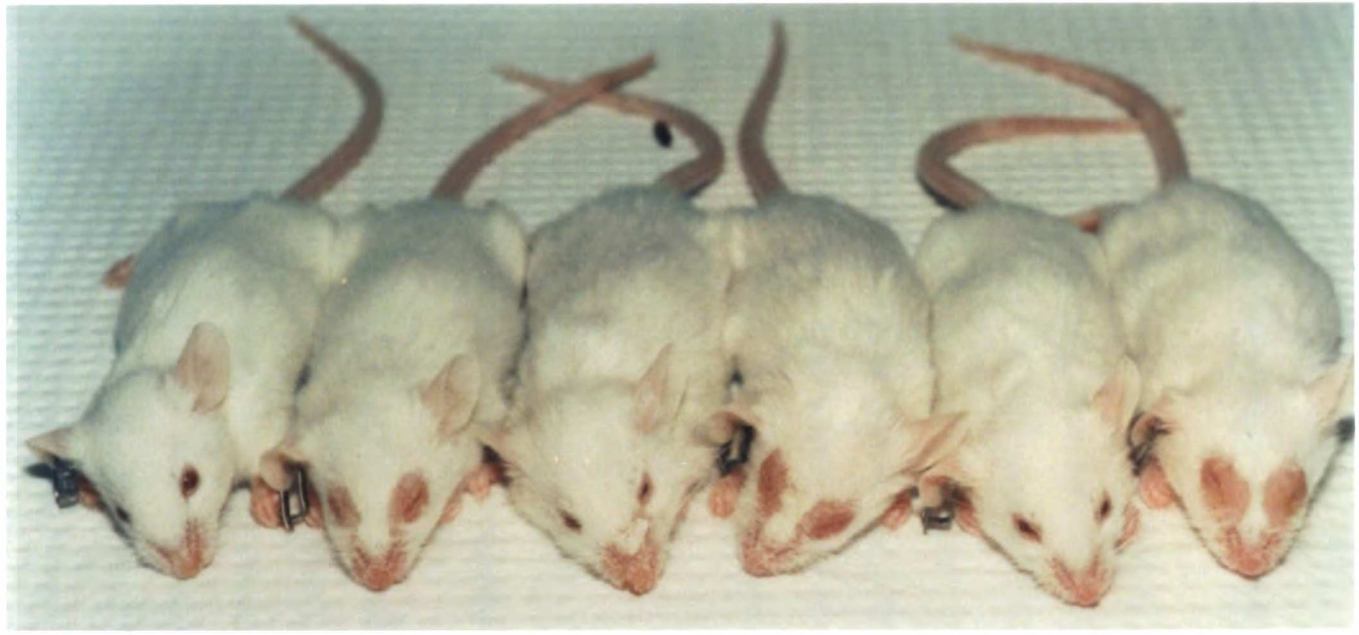
Figure 4.10 ATRA prophylaxis

Six homozygous littermates of line 565 received subcutaneous 21-day timed-release pellets when they were 15 days old. They are photographed at age 6 weeks. Those that received ATRA pellets (+) have developed much less of the facial hyperplasia that is characteristic of this line than those that received placebo pellets (-). Compare also to Figure 4.2 (b) and (c).

Figure 4.10
ATRA prophylaxis



ATRA + - + - + -

due to the extreme papillomatosis (Figure 4.11c). Histologically, the lesions resembled the corrugated regions of the skin of *PMLRARα* mice (Figure 4.11e).

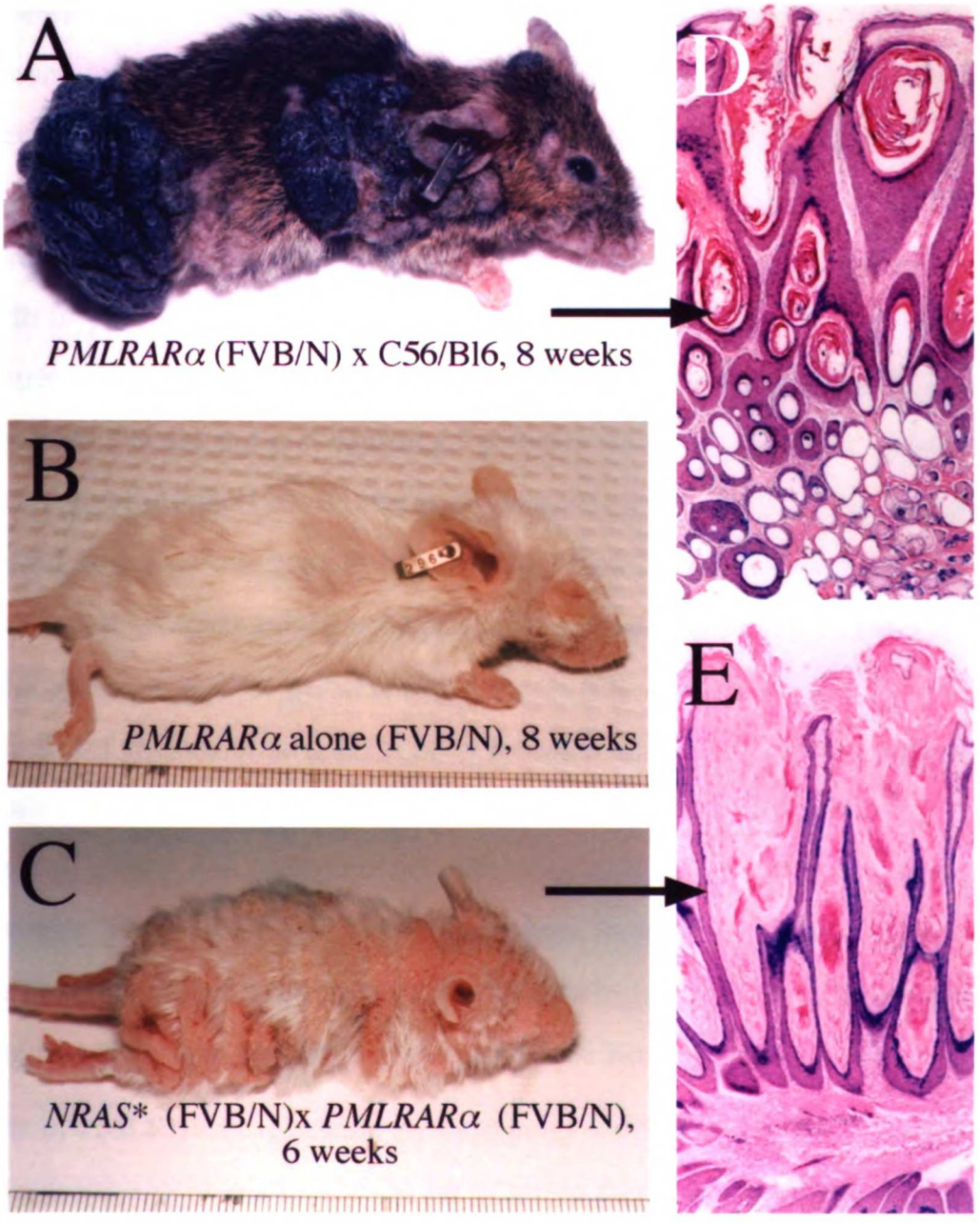
C57/Bl6 mice do not seem to be less susceptible to *PMLRARα*-driven papilloma formation

In an attempt to minimize the impact of the skin lesions on analysis of the hematopoietic phenotype, we crossed mice from two *hMRP8-PMLRARα* transgenic lines (506 and 556) to inbred C57/Bl6 mice, which are generally less susceptible to skin tumor formation than are FVB/N mice (Hennings 1993). The crosses were only followed for two generations: the progeny rapidly developed substantial lesions (Figure 4.11a), which made them impractical to maintain. The histology of the lesions resembled those seen in the inbred FVB/N background (Figure 4.11d).

Figure 4.11 Crosses to C57/Bl6 and *NRAS accelerate tumorigenesis**

The progeny of crosses between FVB/N *hMRP8-PMLRARα* transgenic mice of line 556 and non-transgenic C57/Bl6 mice (a) or FVB/N *hMRP8-NRAS** mice (c) develop more extreme skin tumors more rapidly than is typical for the parent FVB/N line 556 mice (b). Histologically the tumors are similar to those seen in line 556 alone. (a) Mouse 292T, an F1 of a FVB/N *hMRP8-PMLRARα* x C57/Bl6 cross, age 8 weeks. (b) Mouse 1296, FVB/N line 556, at age 8 weeks. (c) Mouse 763, an F1 of a FVB/N *hMRP8-PMLRARα* x FVB/N *hMRP8-NRAS** cross, at age 6 weeks. (d) Tumor from (a) at 20X original magnification. (e) Section of skin from (c) at 20X original magnification.

Figure 4.11
Crosses to C57/Bl6 and *NRAS** accelerate tumorigenesis



Discussion

The skin abnormalities seen in *hMRP8-PMLRARα* mice were consistent with reported patterns of *hMRP8* expression

The pattern of skin abnormalities seen in the *hMRP8-PMLRARα* mice were consistent with reported patterns of *hMRP8* expression, suggesting that they were indeed caused by the transgene. In normal epidermis, Mrp8 has been seen in pilosebaceous units (Gabrielsen 1986; Brandtzaeg 1988); expression in interfollicular epidermis was limited to inflammatory conditions and disease states (Gabrielsen 1986; Wilkinson 1988). The first abnormality to become apparent in the transgenic mice was altered hair growth in young pups, possibly reflecting delayed differentiation of hair-producing cells in the pilosebaceous units. Later, inflammation from insertion of the ear tag and continued irritation from the tag itself at this site might have stimulated continued transgene expression in the area, and led to the rapid and vigorous growth of tumors at these sites. Similarly, bite and experimental wounds might upregulate transgene expression in the interfollicular as well as pilosebaceous epidermis.

Based on studies of long-term label retention, cells in the bulge region of the hair follicle have been hypothesized to be self-renewing stem cells, capable of giving rise to committed sebaceous or keratinocyte precursors (Cotsarelis 1990). Expression of the transgene in such stem cells might deregulate their proliferation or inhibit the differentiation of their progeny, giving rise to lesions combining sebaceous and keratinocyte features.

Effects of PMLRARa expression on keratinocyte differentiation

Immunohistochemistry should be of value in examining the extent to which the abnormal histology in the skin of PmlRarα transgenic mice indicates altered follicular and/or keratinocyte differentiation. We have

initiated studies to analyze the expression of differentially regulated genes in the epidermis of the transgenic mice. The expression of pairs of keratin genes in epidermis is tied to the differentiation state of the cells: K5 and K14 are expressed in basal cells, K1 and K10 are expressed in suprabasal cells, and K6 and K16 are suprabasally expressed in wound healing (Fuchs 1994). In the transgenic skin, a pilot study suggested that K5 and K14 were expressed in suprabasal cells (data not shown). Further analysis of keratin genes, including keratins 1, 5, 6, 10, 14 and 16, and other keratinocyte differentiation markers, such as filaggrin, may help to determine whether there is a consistent alteration in the differentiation of the transgenic keratinocytes.

Lesions in the *PMLRARα* mice resemble retinoid deficiency states

Human vitamin A deficiency states have long been recognized to include marked skin changes "analogous histologically to the pathologic changes in the eye and other tissues of animals and man following deprivation of fat-soluble vitamin A", which precede the development of ophthalmic lesions (Frazier 1931). Generally, the deficiency is marked by hyperkeratosis; in particular, a condition known as phrynoderma is associated with accumulation of keratinaceous material that blocks and disrupts follicular ducts (Wechsler 1979; Nakjang 1988; Rook 1992). These changes seen in vitamin A deficiency were similar to the hyperkeratotic changes seen in *PMLRARα* mice, which suggests that expression of *PMLRARα* renders epidermis effectively deficient in vitamin A--consistent with a dominant negative action of PmlRarα against Rarα.

Vitamin A toxicity is also marked by follicular hyperkeratoses of the skin, although their etiology appears somewhat different: stimulation of mitotic activity throughout the skin results in epidermal thickening and accumulation of keratin marked by parakeratosis, the retention of nuclei in

keratinocytes of the stratum corneum (Jarrett 1980; Rook 1992). The general lack of parakeratosis in the lesions of the *PMLRARα* mice and the prophylactic effects of retinoic acid treatment argue against the hypothesis that PmlRarα acts as a hyperactive Rarα in the skin.

PMLRARα expression does not simply recapitulate dominant negative *RARα* expression in skin

The transgenic mice bearing *PMLRARα* developed hyperplastic skin lesions dominated by keratinaceous whorls and cysts. These papillomas contrast with the hypoplastic epidermis resulting from dominant negative *RARα* expression in basal keratinocytes (Saitou 1995). Suprabasal expression of another dominant negative *RARα* construct did not change the thickness of the epidermal strata, but there was impairment of barrier function due to diminished formation of lipid lamellar bodies in corneocytes (Imakado 1995). There was no evidence of diminished barrier function in the *PMLRARα* transgenic mice; they maintained normal hydration, weight, and activity even when the lesions covered a majority of their skin surface area.

While it remains formally possible that the differences between the epidermis of *PMLRARα* mice and the dominant negative *RARα* mice were due to differential expression of the transgenes, it more likely reflects significant variation in the mode of action of the transgenes themselves. A preliminary abstract discussing *PML* knockout mice suggested that Pml has tumor suppressor activity in the skin (Wang 1996). While PmlRarα probably does exhibit dominant negative Rarα activity on some promoters, a dominant negative inhibition of Pml activity in the skin may also be important, as could novel activity of the fusion gene.

Wounding and papilloma formation

Additional analysis of transgene expression by immunohistochemistry should help to clarify the relationship between transgene expression, inflammation, and tumor formation: if the transgene were only expressed in conditions of inflammation, papilloma formation could be a single step process once the transgene is turned on; if the transgene were normally expressed throughout the epidermis, it would imply that inflammation cooperates with *PMLRARα* to bring about papilloma formation.

Such cooperation would not be unprecedented: mice carrying a v-jun transgene developed dermal fibrosarcomas following full-thickness wounding of the skin, suggesting that elements of the wound repair process promoted tumor formation (Schuh 1990). PmlRarα might have a similar affect on some of the same pathways as the v-jun transgene, as it can stimulate AP-1 activity in the presence of retinoic acid (Doucas 1993). If sufficient retinoic acid were present, it is possible that PmlRarα expression might prime epidermal cells for a tumorigenic response to wound repair much as v-jun primed dermal fibroblasts.

***NRAS* cooperates with *PMLRARα* to accelerate generalized papillomatosis**

The extreme papillomatosis seen in *PMLRARα/NRAS* transgenic mice demonstrates that activated *NRAS* can cooperate with *PMLRARα* to accelerate papilloma formation in the skin. This cooperation is consistent with data from the recent abstract by Wang et. al. in which *PML -/-* mice were more susceptible to skin tumors after DMBA treatment (Wang 1996); DMBA treatment can yield activating mutations in *RAS* family genes (Chakravarti 1995). This was in contrast to the bone marrow, which did not notably differ from the *PMLRARα* transgenic parents. These results could indicate that

NRAS can accelerate epidermal papillomatosis by a pathway that is not active in developing granulocytes; more likely, however, it reflects the inability of the *HMRP8-NRAS* expression to alter granulopoiesis by itself or in cooperation with *PMLRARα*.. These results demonstrate that activation of *RAS*-mediated signalling pathways affects papilloma formation and could potentially have a similar affect on malignant progression in promyelocytic leukemia.

Potential lessons from papilloma formation in *hMRP8-PMLRARα* transgenic mice

The follicular carcinoma seen in the tumor from mouse 556 does not have a direct counterpart in humans. Nonetheless, if malignant progression occurs at a reasonable frequency in the papillomas seen in the *PMLRARα* mice, these lesions could yield information of value regarding tumor progression more generally: for example, through the identification of novel oncogenes that cooperate with *PMLRARα* in tumorigenesis. It should also be noted that while human keratoacanthomas traditionally have been considered benign lesions without metastatic potential (Pinkus 1981; Rook 1992), recent reports have challenged this view, documenting cases in which primary lesions and metastases that had histology characteristic of keratoacanthoma (Hodak 1993). Whether themselves malignant or not, the murine papillomas may also offer insights into tumor progression in epidermal squamous cell carcinomas.

More directly, however, they offer a window into the actions of *PMLRARα*, particularly as contrasted to transgenics made with dominant negative *RARα* . Comparisons of differentiation-regulated genes in these two systems may help distinguish the pathways by which the different transgenes act. Crosses to mice lacking *PML* or *RARs* could also help distinguish

between dominant negative and gain-of-function actions of *PMLRARα*; the latter would be expected to increase the severity of phenotypes in mice bearing the fusion gene over that seen in the deficiency state alone.

Chapter 5

Some additional abnormalities observed in *MRP8-PMLRARα* transgenic mice: respiratory distress, keratinizing squamous metaplasia, and lymphoid malignancies

Introduction

In addition to the very consistent granulocytic and epidermal changes seen in the *MRP8-PMLRARα* mice, we have seen other abnormalities that are less obviously related to transgene expression. Some of these abnormalities may be caused by the transgene and some may be coincidental, but they have not been evaluated sufficiently to determine which is the case. A few of these abnormalities are briefly documented in this chapter because they may reveal further insights into PmlRarα activity, or because they might be of concern to investigators working with these mice. Among these changes, susceptibility to a respiratory distress syndrome is probably the most important, because it has a substantial impact on the life expectancy of transgenic mice expressing the highest levels of the *PMLRARα* transgene. Many female transgenics had difficulty raising their pups, and this may be related to keratinizing squamous metaplasia of the oropharyngeal epithelia and preputial and clitoral glands, and perhaps the harderian glands. Finally, several *hMRP8-PMLRARα* transgenic mice developed lymphoid malignancies, which might indicate that *PMLRARα* is expressed and initiates oncogenesis in lymphoid cells.

Respiratory Distress

hMRP8-PMLRARα mice frequently develop respiratory distress

In lines expressing high levels of the *PMLRARα* transgene, mice frequently developed a syndrome characterized by respiratory distress: they became hunched, appearing to pant while breathing heavily, and looked cyanotic. Because they appeared to be in great distress, the animals were routinely euthanized at the onset of breathing difficulties. We have not seen a comparable phenomenon in our non-transgenic mice.

The syndrome was rarely seen in animals under one month of age, but was frequently seen thereafter. In the high-expressing transgenic line 556, euthanasia for respiratory distress was the leading cause of death, accounting for 40% percent of the mice (problems relating to the skin phenotype accounted for 35%, while leukemias and experiments accounted for most of the rest). Euthanasia in response to respiratory distress was also the leading cause of death in line 565 homozygotes; a few cases have occurred in lines 553 and 506. While occuring with high frequency, respiratory distress does not appear to occur in clusters within single cages, and it does not occur in recipients of transgenic bone marrow transplants, even when the donor animal was showing signs of illness; these characteristics argue against an infectious cause.

Pathology of respiratory distress

Pathological study of the animals has not revealed a cause for the syndrome. The distressed mice typically had papillomatous lesions on their muzzles, but no mechanical blockage of the respiratory tract could be demonstrated when probed directly or examined in histological sections. We cannot rule out a functional blockage, such as might occur if the external

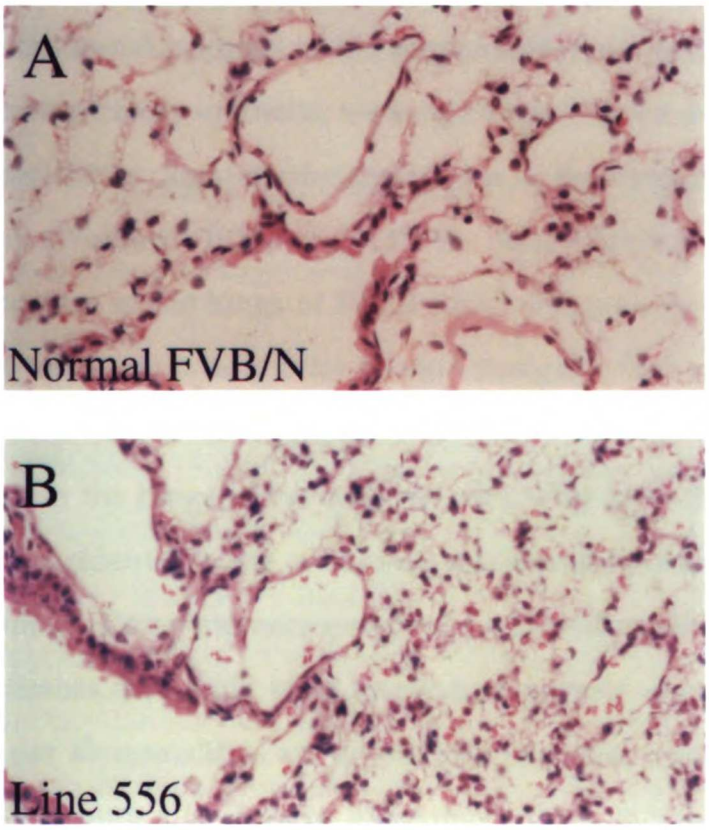
nares collapsed upon inspiration, yet were seemingly open when probed. In the presence of the syndrome, blood and bone marrow counts and smears were consistently unremarkable. There was no profound anemia and no change in circulating leukocytes suggestive of infection. The organs grossly appeared normal. Histologic sections of spleen and bone marrow were normal.

The lungs of the mice which suffered respiratory distress show some subtle changes, including a generalized hypercellularity in the lung parenchyma and dilated lymphatic vessels (Figure 5.1b), which could signal generalized inflammation or perhaps extramedullary hematopoiesis. We can further distinguish among these possibilities by performing lung function studies on living, symptomatic mice; weighing lungs at necropsy to test for edema; and carefully preparing lung specimens for histology. Perfusion fixation of the lungs combined with inflation yields tissues that are far more informative to pathologists and can help to distinguish between a primary hyperplasia of the lung parenchyma, hyperleukocytosis in the vasculature, and extramedullary hematopoiesis.

Figure 5.1 Lung pathology of *PMLRARα* mice in respiratory distress

The lung parechyma of *PMLRARα* mice in respiratory distress (b) has a hypercellular appearance in comparison to the lung of a non-transgenic mouse (a). Many myeloid cells are present in the transgenic lung. (a) FVB/N mouse, age 6 weeks. (b) Mouse 1886, line 556, age 6 weeks. H&E, 160X original magnification.

Figure 5.1
Lung pathology in respiratory distress



Relationship between respiratory distress and transgene expression

Although we have not looked for transgene expression in the lung, there are some reasons to suspect a link between the transgene and lung pathology. The *hMRP8* promoter is not known to drive expression specifically in the bronchi or lung parenchyma, but Mrp8 has been reported in squamous cell carcinomas of the lung (Wilkinson 1988), suggesting that *hMRP8* could drive expression in lung tissue under some circumstances. Mrp8 has also been seen in nasopharyngeal epithelia (Brandtzaeg 1988; Wilkinson 1988), which lines the initial portions of the respiratory tract. If the transgene were expressed in respiratory epithelia, we might expect to see some changes: in vitamin A deficiency, keratinizing metaplasia of the respiratory epithelia is a major feature (Wolbach 1925)(Chytil 1996). Keratinization does not appear to be a major feature in the lungs of *PMLRARα* transgenic mice in respiratory distress, but there might be subtler related changes which are not immediately apparent.

If changes in the lungs of the cyanotic mice were later determined to be secondary or incidental to the syndrome, additional investigations may be worth pursuing. Complete necropsies might identify problems in non-respiratory tissues that could yield respiratory distress as a terminal event. Cardiovascular abnormalities are an obvious example, and a lung specimen from one sick mouse also showed abnormally thick connective tissue in a cardiac valve (data not shown). Defects in the cardiopulmonary structures were commonly seen in mice deficient in *RAR*s, and if there were some embryonic expression of *PMLRARα*, it might interfere with retinoid dependent organogenesis (Mendelsohn 1994).

Ruling out an infectious cause may be even more important than finding a mechanical or developmental problem, both because it might be preventable, and because it might reveal immunodeficiency due to expression of the transgene. We have not looked to see whether the animals developed fevers or other clinical signs of infection, and while we did not see obvious signs of infection in peripheral blood or bone marrow, we did not specifically attempt to rule this out. Blood and other tissues from sick mice could be cultured for bacteria and viruses, particularly if the *PMLRARα* transgenic animals were shown to be functionally neutropenic or monocytopenic.

Keratinizing Squamous Metaplasia

Some transgenic females have trouble raising their litters

Many female *hMRP8-PMLRARα* transgenic mice were unable to raise their pups to weaning age. The pups appeared normal at birth, but rapidly lost ground compared to pups born to non-transgenic mothers. Runting was often apparent within a week after birth, and, if not fostered to non-transgenic mothers, the pups would have died before weaning. The problem was transgene dose-dependent: it was most common in females of the high-expressing transgenic line 556, while females of lines 565 or 553, when homozygous for the transgene, often could not raise their pups, but hemizygous females of the same lines rarely had any problems raising their litters.

These problems were not due to any lack of attentiveness on the part of the mother: the transgenic females, like the FVB/N mice from which the transgenic mice were made, were very attentive to their pups (and so were the males). The pups made appropriate efforts to nurse, and grew normally if

fostered. In some cases, the mammary glands of the transgenic females became enlarged, similar to what is seen when a nursing litter is removed from their mother. In the latter case, the glands become engorged with milk, and mastitis is common. In the transgenic females, mastitis was seen occasionally, although our records are inadequate to determine if there was a statistically significant increase in mastitis among them. Since there did not seem to be any problems with the maternal instincts of the mothers or the nursing ability of the pups, the most likely explanation was an insufficient milk supply to the pups: the enlarged mammary glands of the nursing females suggest that blocked milk ducts could be at the root of the problem. We did not sacrifice the breeding females to collect tissue samples, so we do not yet have pathological evidence for this hypothesis. By analogy with other tissues, however, keratinizing metaplasia of the milk ducts is a likely candidate for the root of the problem.

Keratinizing squamous metaplasia

Keratinizing squamous metaplasia was first described by Wolbach and Howe in their pioneering study of vitamin A deficiency in rats (Wolbach 1925). They took animals that had been raised on a normal diet, and switched them to diet deficient solely in vitamin A. The major hallmark of the pathologies they documented in the mouse was a hyperplasia of secretory epithelia, including respiratory epithelia, the lining of the urinary tract, salivary glands, male genitourinal structures and accessory glands, and harderian glands of the eye. In those tissues, normal epithelium was replaced by stratified layers of keratinizing squamous cells.

Using the tissue distribution of Mrp8 as a guide, we might expect to see keratinizing metaplasia where PmlRarα is expressed, such as nasopharyngeal,

vaginal, and cervical epithelia, if PmlRarα were acting as a dominant negative Rarα. The oral epithelium of the transgenic mice developed many small white keratinaceous cysts, giving it a speckled appearance (Figure 5.2a). These generally did not seem to inconvenience the animals, but infection of these structures may have led to the frequent abcesses seen at the edges of the mouths of line 556 mice (Figure 5.2b). We did not note any unusual keratinization of vaginal or cervical epithelia; however, papillomas did occur frequently on the vulvas of the female mice, and some of those might have initiated from vaginal epithelia rather than the epidermis.

Keratinization of the clitoral and preputial glands

The most profound degree of keratinization, however, occurred in glands which are unique to rodents and therefore were not included in previous surveys of *MRP8* expression. The male preputial gland and female clitoral gland are homologous accessory genital glands that are important for mating and dominance behavior in mice (Ago 1995). These sebaceous glands have stratified squamous epithelial ducts leading to the genital surface (Figure

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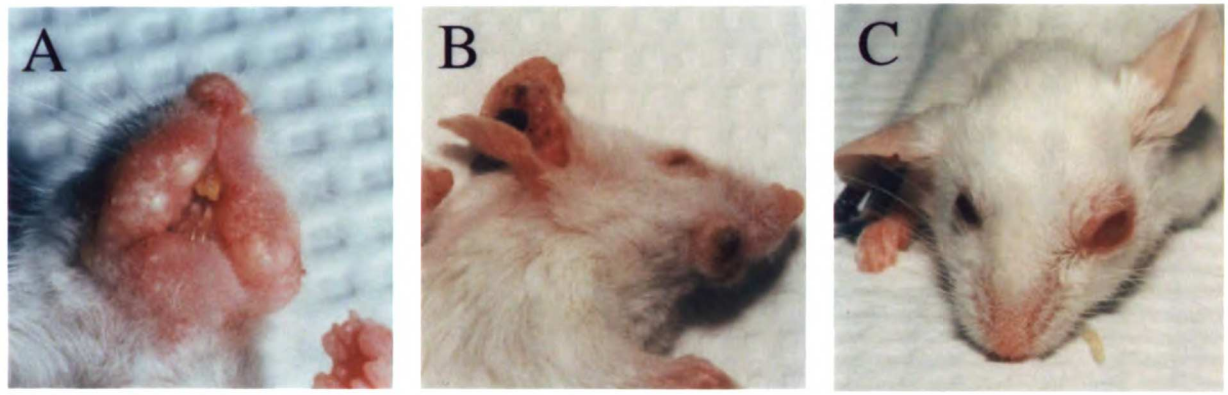
Figure 5.2 Keratinizing squamous metaplasia in *PMLRARα* mice

Keratinization of several non-epidermal tissues has been seen in *PMLRARα* mice. Keratinaceous cysts appear as white speckles in the oral epithelia of *PMLRARα* transgenic mice. (a) Lesions like these at the edge of the mouth continue into the epithelium lining the mouth. (b) The lips are common sites of abcesses like this (arrow). (c) Eye protrusion like this may be related to hyperplasia of the harderian gland. (a) Mouse 596, line 506, age 8 months. (b) Mouse 771, line 556, age 3 months. (c) Mouse 656, line 506, age 3 months.

Figure 5.2
Keratinizing Squamous metaplasia



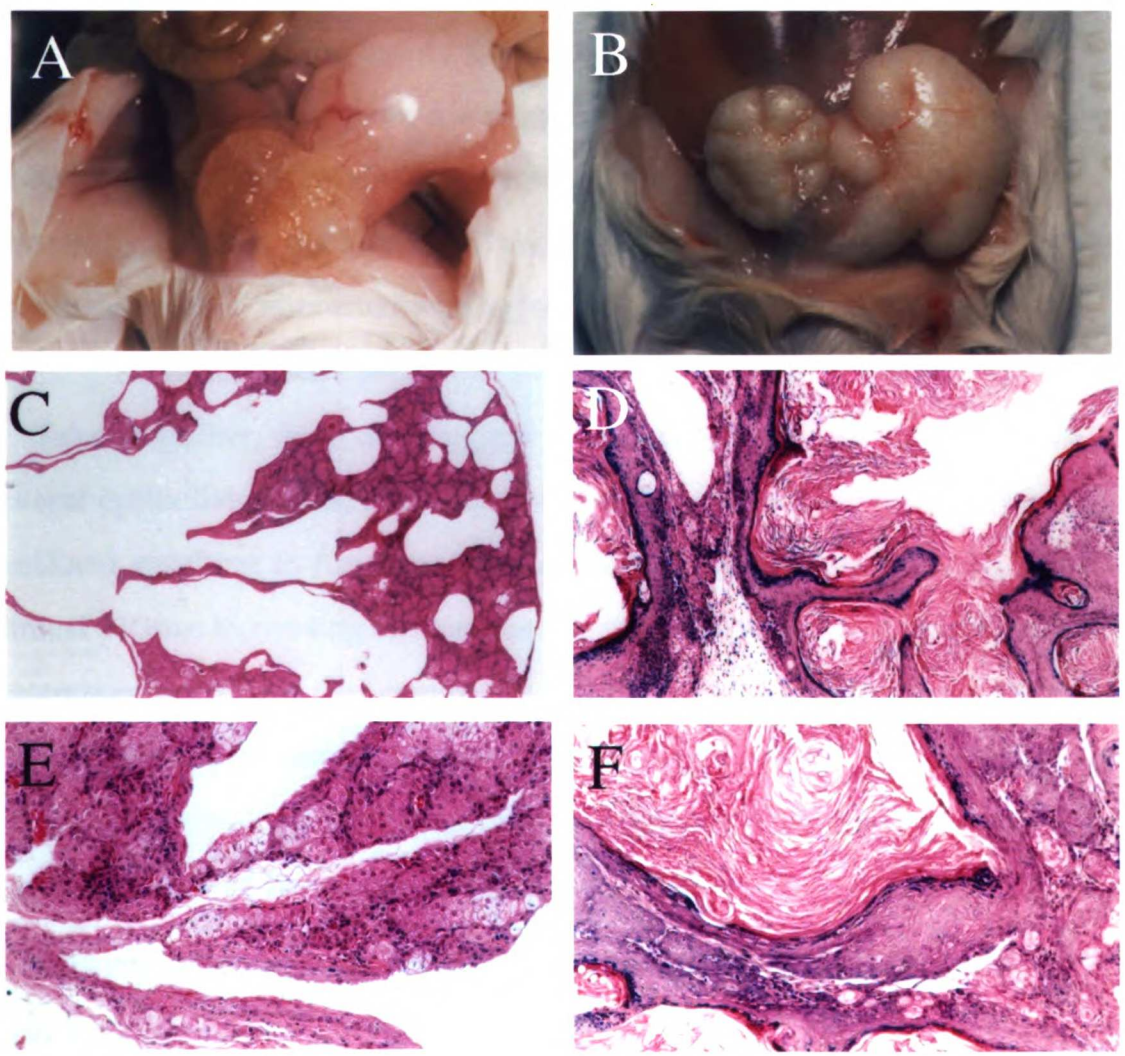
5.3c,e) (Stolte 1993). Based on their histology, then, they are likely sites for *MRP8* expression.

Normally these glands are soft, translucent structures lying subcutaneously anterior to the genitals (Figure 5.3a)(Green 1966). In a substantial proportion of the *PMLRARα* transgenic mice these glands became visibly enlarged, particularly in older mice. Occasionally they became sites of inflammation and abcesses. Grossly, the enlarged glands were firm, white and opaque (Figure 5.3d). Microscopically the glands were almost completely taken over by keratization (Figure 5.3e), with the few remaining sebaceous cells relegated to a thin rim outside hyperplastic epithelia surrouding irreglar keratin whorls (Figure 5.3f). Small foci of hyperplasia are common in the preputial glands of aged mice (Stolte 1993), but the extreme degree of hyperplasia and keratinization of the glands in the transgenic animals fell outside the normal range. Therefore, the changes seen in the glands of the transgenic mice most likely represent keratinizing metaplasia due to *PMLRARα* expression.

Figure 5.3 Keratinizing squamous metaplasia of the preputial gland

The normal preputial glands in the male mouse are a translucent yellow (a), and are filled with sebacous cells and ducts lined with stratified squamous epithelia a couple of cells thick (c,e). Firm, white, opaque glands like these (b) are frequent in the *PMLRARα* transgenic mice. The glands are full of hyperplastic epithelia and keratin (d) and the normal sebaceous tissue is hardly visible (e). (a,c,e) 2 month old FVB/N. (b) Mouse 1257, line 565, age 8 months. (d,f) Mouse 2016, line 565, age 2 months. Original magnifications of H&E stained tissues were 20X (b,e) and 80X (c,f).

Figure5.3
Keratinizing metaplasia of preputial glands



FVB/N

Line 565

Retroorbital keratinizing metaplasia?

Another tissue that may represent a site of keratinizing metaplasia is the Harderian gland, which is another tissue that has not been analyzed for the presence of Mrp8: it is an accessory lacrimal gland that is rudimentary in humans. Occasionally one eye of a transgenic animal became inflamed and bulged out from its socket due to enlargement of retroorbital tissues (Figure 5.2c). In some cases, there was a distinct retroorbital abcess, but not always. Due to the nature of the lesion, the animals were routinely euthanized. Sometimes a yellowish gland a few millimeters in diameter was distinguished, which might have been an enlarged harderian gland. We have not confirmed the identity of the structure because the small size of the tissue, even when abnormally enlarged, complicated preparation of histological specimens.

Taken together, these data suggest a pattern of squamous metaplasia in several epithelial tissues, consistent with a dominant negative effect of PmlRarα resulting in functional retinoid deficiency in those tissues. The clinical picture in nursing female transgenics may be due to a keratinizing squamous metaplasia that eventually blocks the milk ducts, leading to engorgement of the mammary glands and starving pups. This hypothesis can be tested by histological study of mammary glands from nursing female transgenic mice of line 556 or line 553 homozygotes.

Lymphoid malignancies and a possible paraneoplastic syndrome

PMLRARα mice develop lymphoid malignancies

Three lymphoid malignancies were documented in the *hMRP8-PMLRARα* mice. While this very small number of cases could represent spontaneous disease, independent of transgene expression, several lymphoid

malignancies have also been seen in the *hMRP8-NRAS* mice, supporting the hypothesis that the *hMRP8* promoter drives transgene expression in cells that can give rise to lymphoid and myeloid derivatives. These cases may therefore be worthy of further investigation as potential effects of *PMLRARα* expression. One of these cases may be worthy of further investigation even in the absence of transgene expression, because it was associated with an ascending paralysis that may have been a manifestation of a paraneoplastic syndrome.

The three lymphoid malignancies occured in mice aged 3-11 months, from three different transgenic lines. In each case the mouse presented with subcutaneous cervical masses while remaining active and in no apparent distress (Figure 5.4a). Unlike the mice with APL, these animals had high white cell counts in their peripheral blood, up to 2×10^8 cells/mL. Lymph nodes were tremendously enlarged, firm and white (Figure 5.4d). Spleens were mildly enlarged and bone marrow was pale; livers were not noticeably enlarged. Smears of blood and bone marrow revealed large numbers of large

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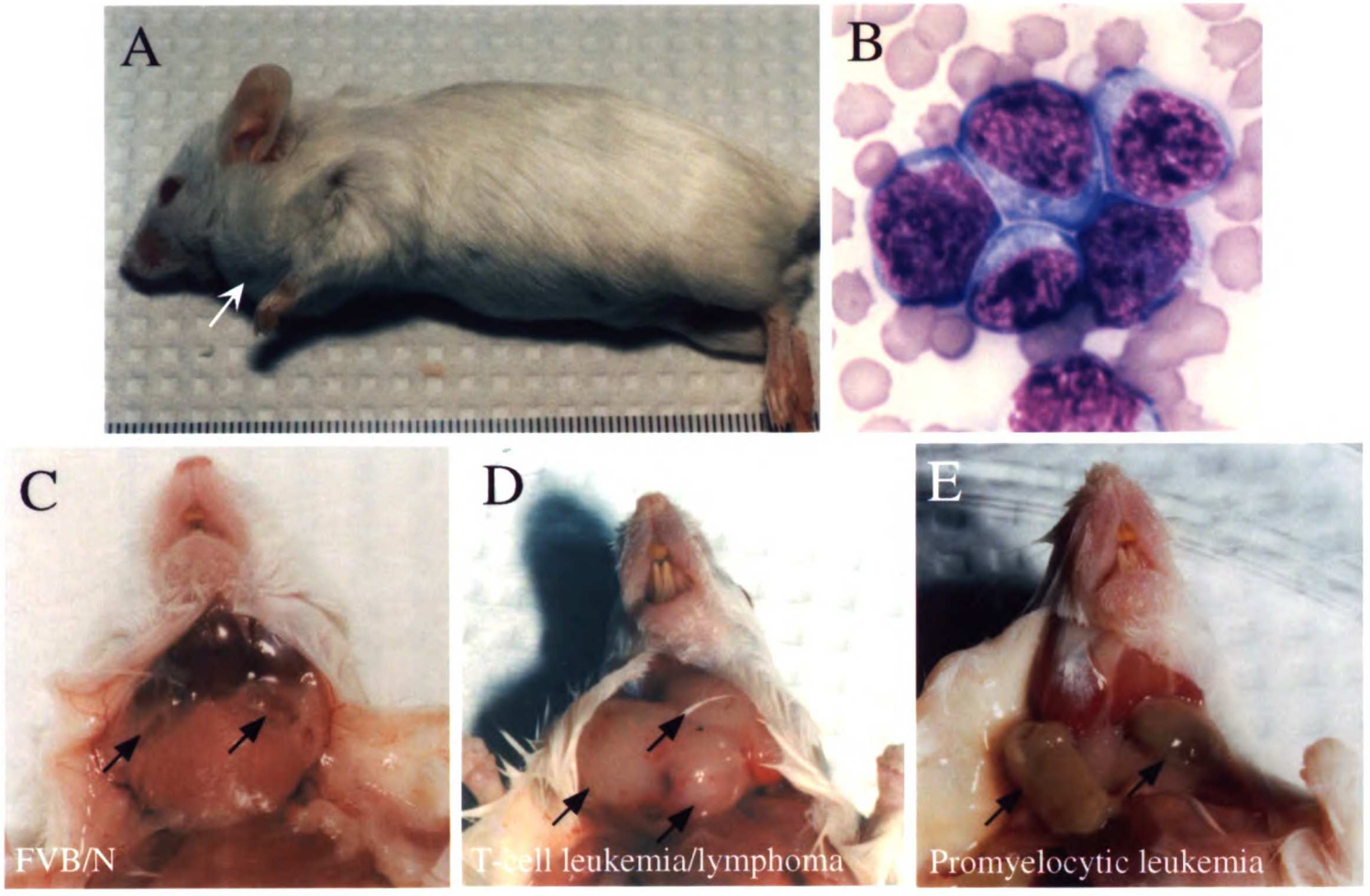
Figure 5.4 Lymphoid malignancies in *PMLRARα* transgenic mice

Three mice presented with cervical lymphadenopathy (a, arrows) in which the enormously enlarged lymph nodes were white (d), in contrast to the smaller green nodes characteristic of mice with APL (e). Normal lymph nodes are barely visible in the connective tissue of the neck (c, arrows). Lymphoblasts were present in the blood and bone marrow (e). (a,b,d) Mouse 91T, line 553, age 12 months. (e) Mouse 394T, first passage of leukemia 1450, line 556. (c) 2 month old FVB/N. (b) Bone marrow smear from mouse 91T. Wright-Giemsa with Azure B, original magnification 400X.

Figure 5.4
Lymphoid malignancy



blasts that had open chromatin in a round nucleus, basophilic cytoplasm, and none of the primary granules characteristic of promyelocytes (Figure 5.4b). By FACS analysis, the marrows were dominated by cells that expressed low levels of the Mac-1 and Gr-1 antigens but had high levels of either the B-cell marker B220 (mouse 10T of line 506) or the T-cell marker Thy1.1 (mouse 1348 of line 556 and mouse 91T of line 553). The pathology and pattern of molecular markers distinguished these lymphoid tumors from the more common promyelocytic leukemias.

We successfully transplanted two of the three leukemia/lymphomas (B-cell from mouse 10T and T-cell from mouse 91T) into unirradiated FVB/N hosts, which developed lymphadenopathy two to three weeks after transplantation; recipients of the third (T-cell from mouse 1348) showed no signs of illness and were euthanized two months after transplantation. At least two of the three lymphoid tumors, therefore, were malignant.

Transgene expression in lymphoid malignancies

The B-cell leukemia/lymphoma was examined for transgene expression. We could not detect PmlRarα by western blot; the results of immunofluorescence were ambiguous. Thus, these leukemia/lymphoma cells were not expressing high levels of the transgene, if indeed it was expressed at all. In considering these results, it should be noted that we were not able to detect expression of the transgene by western blot in bone marrow from several transgenic lines which did show effects of transgene expression, including the development of APLs. Low levels of transgene expression may be compatible with oncogenic effects.

The *PMLRARα* transgene might be involved in leukemogenesis by being expressed directly in lymphoid precursors, by changing the fate of myeloid

precursors, or affecting a very early multipotent progenitor cell. Acting directly in lymphoid cells, *PMLRARα* could promote growth: when expressed in a MOLT-4 human T-cell leukemia cells, *PMLRARα* had effects consistent with growth acceleration, and ATRA had opposite effects (Maeda 1996). *PMLRARα* might change the developmental fate of a precursor cell from myeloid to lymphoid: dominant negative *RARα* was able to alter fates within the myeloid lineage (Tsai 1992), and *PMLRARα* might similarly alter the fate of an earlier precursor cell. *PMLRARα* could also be acting on a very early multipotent progenitor cell: dominant negative *RARα* was able to block the differentiation of very early hematopoietic progenitor cells which were otherwise capable of giving rise to myeloid, lymphoid and erythroid cells (Tsai 1994); if the *hRMP8* promoter drove expression in such an early cell, *PMLRARα* expression might be lymphomagenic.

A paraneoplastic-like syndrome was associated with the 10T leukemia/lymphoma

Recipients of transplants of the 10T leukemia/lymphoma developed a syndrome of ascending paralysis resembling rare paraneoplastic syndromes seen in human cancer patients. The animals generally appeared healthy until the onset of the paralysis one to two weeks after transplantation (Figure

5.5a). The paralysis initially was manifest as weakness of the tail with decreased tail reflexes; later, weakness of the hindquarters was accompanied by spasticity of the hind legs as the animals lost use of them. If allowed to progress, the paralysis eventually included the forelimbs, and the mouse died shortly thereafter. The unusually clear and progressive ascending pattern of this paralysis did not suggest routine leukemic meningitis; it more closely resembled human paraneoplactic syndromes.

Human paraneoplastic syndromes include a number of neurological changes, most commonly associated with the onset of autoimmunity to neurological antigens (Darnell 1996). In subacute necrotizing myelopathy, one of the rarest paraneoplastic syndromes, progressive necrosis of the spinal cord results in an ascending paralysis that is often fatal (Ojeda 1984). The myelopathy has been associated with many solid and hematologic tumors, particularly lymphoid malignancies, and occurs in the absence of direct invasion by the tumor of the meninges or nervous system parenchyma. The spinal cord necrosis instead appears to be the result of tumor cells producing

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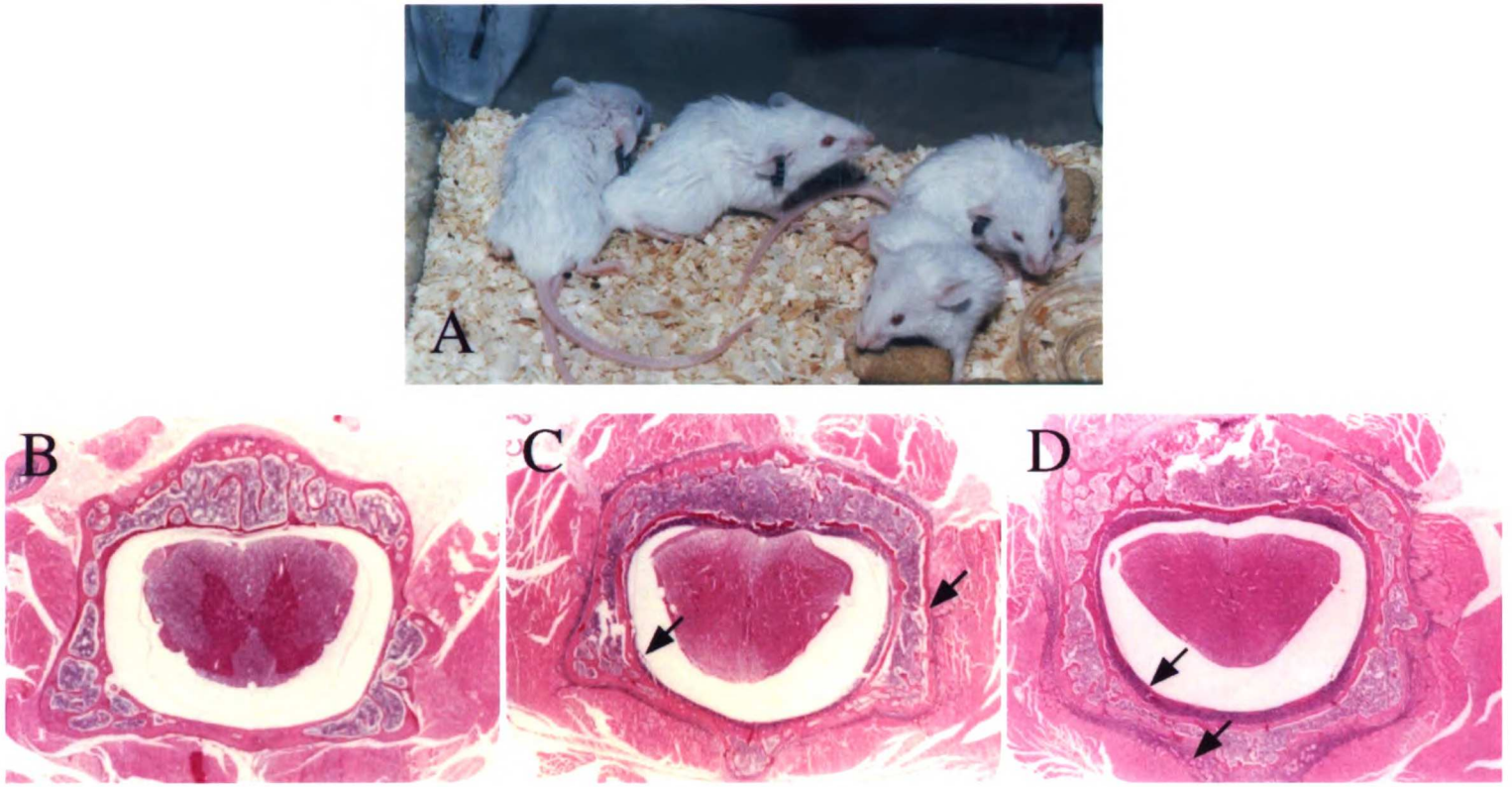
Figure 5.5 Ascending paralysis and lymphocytic meningitis

These 8 week old FVB/N mice received transplants of bone marrow from mouse 10T. 14 days after transplantation they appeared normal; 15 days after transplantation they were paraplegic (a). The tumor invaded the meninges of the brain and spinal cord and surrounding tissues (c,d, arrows). (b) Thoracic spine of 6 week old FVB/N mouse. (c,d) Thoracic spines of mice 7 days after transplantation of leukemia 10T, passage 9. Mouse 1668 (c) received 10^5 cells and had weak tail curling reflexes. Mouse 1666 (d) received 10^6 cells and was quadriplegic and moribund. (b-d) H&E, original magnification 20X.

Figure 5.5
Paraplegia and leukemic menigitis



antigens that are normally expressed exclusively in the immunologically privileged regions of the central nervous system (Darnell 1996). Outside their protected environment, such antigens can stimulate a toxic immune response.

In recipients of transplanted 10T leukemia/lymphoma, the tumor cells invaded the meninges, surrounding tissues, and sometimes the brain or spinal parenchyma itself, and could occasionally be found in the cerebrospinal fluid of the ill mice (Figure 5.6 c,d). Large-scale necrosis of the sort seen in necrotizing myelopathy was absent, although occasionally gliosis and other signs of inflammation were present. The pathological findings in the paralyzed mice were therefore not consistent with a diagnosis of necrotizing myelopathy. Neither were they typical of leukemic meningitis, in which the clinical symptoms are more generalized, without a specific rostral-caudal progression.

The most definitive test for immunological mediation of the syndrome would be passive transfer of the syndrome to other mice. Myasthenia gravis has been experimentally induced in mice following injection of cell-free sera from MG patients (Burges 1994), demonstrating the feasibility of such an undertaking. A large quantity of filtered cell-free sera would be necessary to permit daily injections of sera into a cohort of mice for several weeks. If the test animals developed neurological abnormalities, further exploration of the pathology of the unusual leukemia/lymphoma-associated paralytic syndrome would be warranted.

Chapter 6
Conclusions

The work reported here demonstrates that *PMLRARα* initiates the development of acute promyelocytic leukemia. It is further capable of initiating keratoacanthoma development in the epidermis and causing keratinizing squamous metaplasia in other epithelial tissues. These results have implications for the mechanisms by which *PMLRARα* interferes with normal cellular differentiation and proliferation.

In bone marrow, epidermis and secretory epithelia, *PMLRARα* expression results in changes that in part resemble those seen in retinoid deficiency or resulting from expression of a dominant negative retinoic acid receptor. In the epidermis, *PMLRARα* expression does not duplicate precisely the effects of dominant negative *RARα* expression. Both dominant negative Rarα and PmlRarα may have dominant negative effects on more than just RAREs, because they can also affect other binding partners of RXR, such as VDR, PPAR, and TR. There is no evidence suggesting that dominant negative Rarα and PmlRarα act differently in regard to other RXR binding partners.

The action of PmlRarα putatively differs from that of dominant negative Rarα in that it also may affect Pml and other proteins associated with NBs. Although these structures have as yet no clearly defined functions, they have been associated with growth control by the presence there of oncogenes, by their changes during the cell cycle, and by their response to signals associated with proliferation. Disruption of these pathways, whether through action via

binding directly to Pml itself or through other NB proteins, could have proliferative effects.

The combination of impairment of differentiation and growth promotion together probably account for most of the effects of PmlRarα. This hypothesis is consistent with recent reports in cell culture (Grignani 1996). Thus, in the epidermis, dominant negative Rarα may block various aspects of keratinocyte differentiation, but PmlRarα might also enhance proliferation, explaining why the transgenic mice expressing dominant negative *RARα* die from lack of epidermal barrier function while mice expressing *PMLRARα* have hyperkeratotic epidermis.

In hematopoiesis, the effects of transgenic expression of dominant negative *RARα* expression have not been tested. Very likely it would also have some differentiation inhibitory effects in the same lineages as PmlRarα, but it might not induce leukemogenesis at the same rate, or at all, because it would not be simulataneously inhibiting growth suppressive activities of Pml. Expression of a dominant negative *RARα* under the control of the hMRP8 promoter would provide an intriguing opportunity to test this hypothesis.

Thus far, our analysis of the mice has not revealed any activities of PmlRarα that are not consonant with a combination of effects on Pml and Rarα pathways. Neither does our analysis rule out any novel effects from the juxtaposition of motifs from the two different proteins. For example, it is perfectly plausible that part of the difference between dominant negative Rarα and PmlRarα effects in transgenic mouse epidermis is due to novel gain-of-function actions of PmlRarα. Further analysis of the *hMRP8-PMLRARα* mice might reveal novel activities of PmlRarα, but in the absence

of a clearer understanding of Pml function, it would be difficult to distinguish them from effects mediated via Pml or NBs.

Some clarification might be found through crossing *hMRP8-PMLRARα* mice and mice bearing *PML* , *RARα*, and/or *RXR* under the *hMRP8* promoter. Coexpression of the full-length genes might overcome dominant negative effects of the fusion gene on Pml or Rarα-dependent pathways, and coexpression of *RXR* could help to minimize dominant negative effects of PMLRARα or RARα on the activity of other RXR-dependent factors, such as TR or VDR.

Assuming some degree of inhibition of both differentiation via Rarα motifs and proliferation via Pml motifs, the *hMRP8-PMLRARα* mice demonstrate that expression of *PMLRARα* alone is insufficient for tumorigenesis. Latency, incomplete penetrance and variable expressivity of hematopoietic and epithelial phenotypes demonstrate that additional changes are needed before the already abnormal cells become truly malignant.

In addition to demonstrating that additional changes are needed to progress to malignancy, the *hMRP8-PMLRARα* mice also provide a tool to study those additional changes. Using genomic hybridization studies, loss of heterozygosity analysis, or proviral tagging, it may be possible to identify and clone some of the genes that cooperate with *PMLRARα* during malignant progression.

Should the analysis of these additional genes lead to promising therapeutic strategies, they can be tested on the transgenic leukemias. The transgenic leukemias provide a general resource for preclinical testing of new therapies for APL, and also a tool to probe the effectiveness of current treatments, such as ATRA and As_2O_3.

The goal of this work was to answer a simple question: does *PMLRARα* play a role in leukemogenesis? Although the question was simple, and the tight association between the translocation fusion gene and the disease made an affirmative answer seem likely, the result was not assured. The answer, however, has become far more interesting than a simple "yes", and holds the promise of many exciting discoveries still to come.

Appendix A:
Materials and Methods

Note: methods discussed in chapter 2, "A *PMLRARα* transgene initiates acute promyelcoytic leukemia", are not duplicated here.

Arsenic treatment

Animals were injected subcutaneously once daily with the indicated quantity of As_2O_3 in PBS at 100µg/mL.

Immunohistochemistry

Animals were perfused with fresh 3.75% paraformaldehyde in PBS. Tissue samples were prepared and processed as described with antibodies to keratins 5 and 14 (Arbeit 1994).

Brdu incorporation

Animals were injected with Brdu and two hours later were sacrificed and tissues prepared as described (Arbeit 1994).

Experimental wounding

Wound clips were placed on the back skin of animals anesthetized per routine procedures (Hogan 1994). After 12 days, the wound clips were removed.

Collection of cerebrospinal fluid

Fluid was collected from the exposed fourth ventricle of anesthetized and immobilized mice using a stereotactic micromanipulator. Cytospins of the fluid samples were prepared as in Chapter 2 for analysis of cellular content.

Appendix B:

Summary of *hMRP8-PMLRARα* transgenic lines and phenotypes

Table B1 summarizes transgene expression and phenotypes observed in the various lines of *hMRP8-PMLRARα* transgenic mice. Out of the original 11 transgenics, 9 founded lines. Mouse 562 showed no phenotypic changes and left no transgenic offspring. Mouse 571 had skin tumors but no offspring. The other 9 mice all had offspring which developed skin tumors: it seems likely that there was some minimal level of transgene expression in these all of those lines. Very few mice were bred of lines 564, 567 and 573 (about a dozen each), as they seemed to have relatively low expression and little phenotype, and they were culled early on; a few more mice were bred of lines 569 or 608, but again, they did not have distinctive features and were not extensively analzyed prior to being culled.

We concentrated on the following lines: 556 had the strongest transgene expression by immunofluorescence and western blot, as well as the clearest hematopoietic and epidermal phenotypes; tail biopsies were generally unnecessary because the litters could accurately be typed and culled at 11 days; however, it was difficult to keep, requiring very close attention since respiratory distress and rapid growth of papillomas limited the lifespan, especially of the males. Line 565 had high transgene expression, moderate marrow phenotype by FACS, a relatively minor degree of skin phenotype, and it could easily be carried in homozygotes; the limited lifespan of the

homozygotes, which typically succumbed to respiratory distress, was generally long enough to permit breeding. Line 553 had low levels of transgene expression and minimal marrow phenotype by FACS, but it was bred early in large numbers, out of which several leukemias emerged; the skin phenotype was not dramatic except in homozygotes. Line 506 had intermediate levels of transgene expression, a moderate hematopoietic FACS phenotype, and a moderate degree of skin phenotype.

Key to the table:

ND--not determined

NA--not applicable (no line was founded)

Median Survival--taken from confirmed transgenics that had died prior to 12/96, and excluding those killed for tissue collection

Homozygotes?--refers to whether or not there were two distinct degrees of skin phenotype, the stronger of which bred true or segregated appropriately in crosses between transgenics of the same line

Western--relative expression as determined by visual comparison on western blot

IMF--relative proportion of cells staining strongly with anti-PML antisera on cytospins of unfractionated bone marrow

FACS changes--refers to altered expression of myeloid differentiation markers; see chapter two for details

APLs--number of promyocytic leukemias observed in the line as of 12/96; see chapter two for details

ALL/Ls--number of acute lymphoid leukemia/lymphomas observed in the line; see chapter 5 for details

Ear tag tumors--virtually all of the transgenics developed at least a small lesion at the ear tag site (such as in figure 4.1b); see chapter 4 for details

Non-ear tag tumors--refers to the overall rate of papilloma development at sites other than the ubiquitous ear tag; see chapter 4 for details

Respiratory distress--cyanosis accompanied by tachypnea was common in line 556 and line 565 homozygotes, but otherwise rare; see chapter 5 and Appendix C

Keratinizing metaplasia--without reference to incidence, indicates whether keratinzed preputial or clitoral glands were seen in the line (detailed records were not kept of this phenomenon); see chapter 5

Nursing problems--when female transgenics of some lines were bred, their pups were runted and died if not fostered; see chapter 5 for details

Table B1: Transgenic Lines, Transgene Expression, and Phenotypes

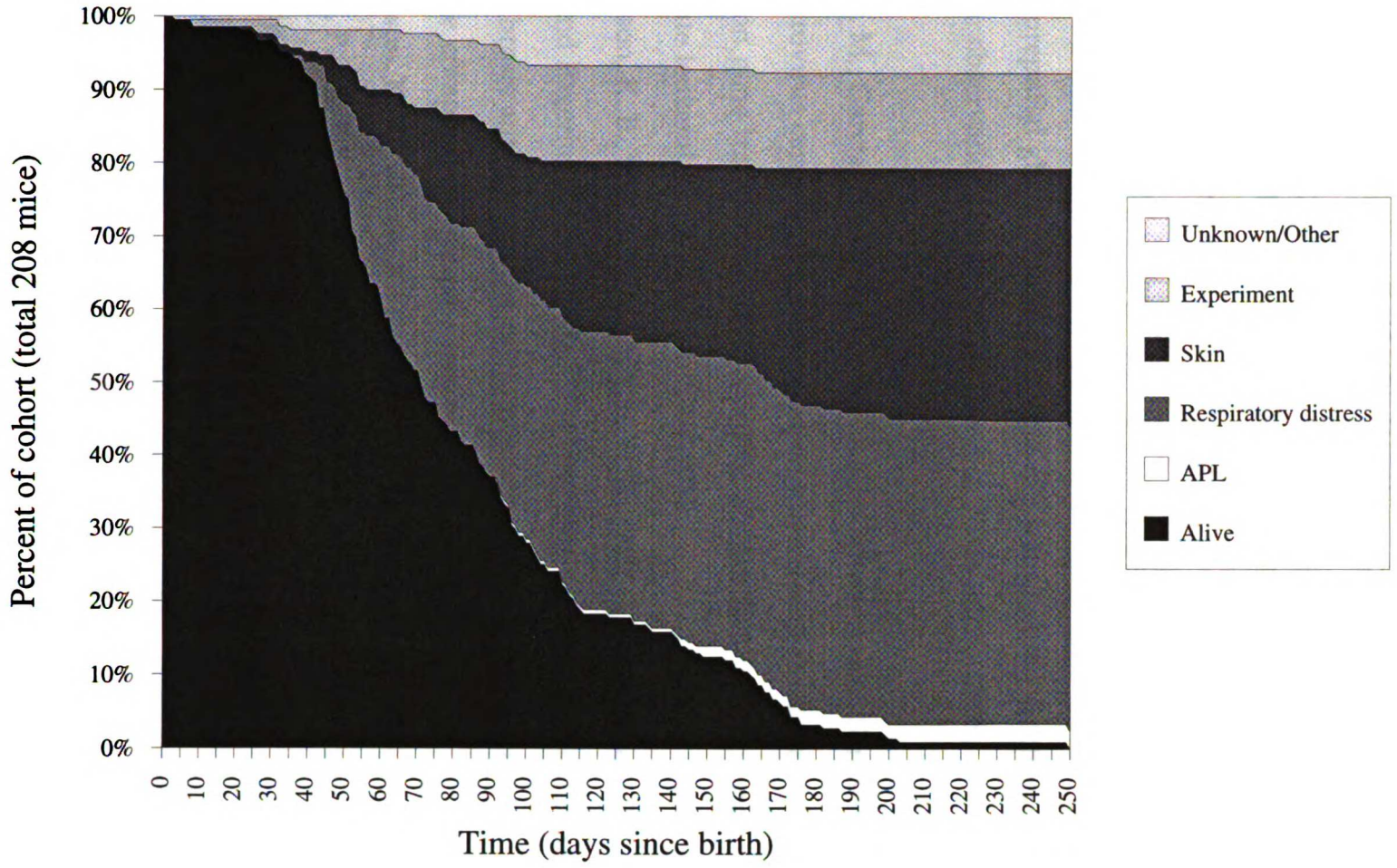
	Line				Marrow Transgene Expression		Hematopoietic Phenotype			Skin Tumors		Miscellaneous Phenotypes		
Original Transgenic	*Founded line?*	*Line kept?*	*Median survival (days)*	*Homozygotes?*	*Western blot*	*Immunofluorescence*	*FACS abnormalities*	*APL cases*	*ALL/L cases*	*At ear tags*	*All other sites*	*Respiratory distress*	*Keratinizing metaplasia*	*Nursing problems*
556	Yes	Yes	79 (189 mice)	Not viable	+++	+++	++	5	1	+	+++	Common	Yes	Yes
565	Yes	Yes	231 (10 mice)	Can reproduce	++	+	+	0	0	+	+/-	Rare	Yes	No
553	Yes	Yes	182 (59 mice)	Can reproduce	+	+	+/-	3	1	+	+	Rare	Yes	No
506	Yes	No	140 (63 mice)	Don't survive to weaning	++	+	+/-	2	1	+	++	Rare	Yes	No
569	Yes	No	165 (11 mice)	ND	+	+	ND	1	0	+	+	None	Yes	No
608	Yes	No	231 (17 mice)	ND	+	+	ND	1	0	+	+	None	Yes	No
567	Yes	No	ND	ND	-	+	ND	0	0	+	-	Rare	Yes	No
573	Yes	No	ND	ND	ND	ND	ND	0	0	+	+	None	ND	No
564	Yes	No	ND	ND	-	+	ND	ND	ND	+	-	ND	ND	ND
571	No	No	ND	ND	+	+	ND	ND	ND	+	+	ND	ND	ND
562	No	No	ND	ND	ND	ND	ND	ND	ND	-	-	ND	ND	ND
Line 553 Homozygotes			ND	-	ND	ND	+	0	0	+	++	Rare	Yes	Yes
Line 565 Homozygotes			76 (49 mice)	-	ND	+++	++	0	0	+	++	Common	Yes	Sometimes
Line 556x565 Transheterozygotes			23 (13 mice)		ND	ND	+++	0	0	+	+++++	Always	ND	ND

Appendix C

Causes of death in line 556

The accompanying chart graphs the cause of death for 208 mice of the high-expressing *hMRP8-PMLRARα* line 556 (Figure C1). The category "experiment" includes those animals that were sacrificed to prepare tissues for analysis; as this group occasionally included animals already showing signs of respiratory distress or with advanced skin lesions, the latter categories are probably slightly underrepresented. The category "skin" includes animals euthanized due to skin lesions that were large enough to interfere with their activities or that were inflammed, infected or bleeding. The category "respiratory distress" includes mice that were euthanized when they became tachypneic, cyanotic, or were panting heavily. Animals diagnosed with murine acute promyelocytic leukemia are listed as "APL".

Figure C1: Causes of Death in Line 556



References

Achkar, C. C., Bentel, J. M., Boylan, J. F., Scher, H. I., Gudas, L. J. and Miller, W. H., Jr. 1994. Differences in the pharmacokinetic properties of orally administered all-trans-retinoic acid and 9-cis-retinoic acid in the plasma of nude mice. *Drug Metab Dispos* **22**: 451-8.

Ago, A., Gonda, T., Kawakami, K. and Sahata, H. 1995. Morphological studies on preputial gland of mice--1. Light and transmission electron microscopic observations of preputial gland in male mice. *Jikken Dobutsu* **43**: 645-9.

Altabef, M., Garcia, M., Lavau, C., Bae, S.-C., Dejean, A. and Samarut, J. 1996. A retrovirus carrying the promyelocyte-retinoic acid receptor PML-RARα fusion gene transforms haematopoietic progenitors in vitro and induces acute leukemias. *EMBO J* **15**: 2707-2716.

Andersson, K. B., Sletten, K., Berntzen, H. B., Dale, I., Brandtzaeg, P., Jellum, E. and Fagerhol, M. K. 1988a. The leucocyte L1 protein: identity with the cystic fibrosis antigen and the calcium-binding MRP-8 and MRP-14 macrophage components. *Scand J Immunol* **28**: 241-5.

Andersson, K. B., Sletten, K., Berntzen, H. B., Fagerhol, M. K., Dale, I., Brandtzaeg, P. and Jellum, E. 1988b. Leukocyte L1 protein and the cystic fibrosis antigen. *Nature* **332**: 688.

Arbeit, J. M., Munger, K., Howley, P. M. and Hanahan, D. 1994. Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. *J Virol* **68**: 4358-68.

Ascoli, C. A. and Maul, G. G. 1991. Identification of a novel nuclear domain. *J Cell Biol* **112**: 785-95.

Benedetti, L., Grignani, F., Scicchitano, B. M., Jetten, A. M., Diverio, D., Lo Coco, F., Avvisati, G., Gambacorti-Passerini, C., Adamo, S., Levin, A. A., Pelicci, P. G. and Nervi, C. 1996. Retinoid-induced differentiation of acute promyelocytic leukemia involves PML-RARalpha-mediated increase of type II transglutaminase. *Blood* **87**: 1939-50.

Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A., Gralnick, H. R. and Sultan, C. 1985. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med* **103**: 620-5.

Biondi, A., Luciano, A., Bassan, R., Mininni, D., Specchia, G., Lanzi, E., Castagna, S., Cantu-Rajnoldi, A., Liso, V., Masera, G., Barbui, T. and Rimbaldi, A. 1995. CD2 expression in acute promyelocytic leukemia is associated with microgranular morphology (FAB M3v) but not with any PML gene breakpoint. *Leukemia* **9**: 1461-6.

Borrow, J., Goddard, A. D., Gibbons, B., Katz, F., Swirsky, D., Fioretos, T., Dube, I., Winfield, D. A., Kingston, J., Hagemeijer, A., Rees, J. K. H., Lister, T. A. and Solomon, E. 1992. Diagnosis of acute promyelocytic leukaemia by RT-PCR: detection of PML-RARA and RARA-PML fusion transcripts. *Br J Haematol* **82**: 529-40.

Bos, J. L. 1989. Ras oncogenes in human cancer: a review. *Cancer Res* **49**: 4682-9.

Brandtzaeg, P., Jones, D. B., Flavell, D. J. and Fagerhol, M. K. 1988. Mac 387 antibody and detection of formalin resistant myelomonocytic L1 antigen. *J Clin Pathol* **41**: 963-70.

Breitman, T. R., Collins, S. J. and Keene, B. R. 1981. Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid. *Blood* **57**: 1000-4.

Breitman, T. R., Selonick, S. E. and Collins, S. J. 1980. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc Natl Acad Sci U S A* **77**: 2936-40.

Bruggen, J., Tarcsay, L., Cerletti, N., Odink, K., Rutishauser, M., Hollander, G. and Sorg, C. 1988. The molecular nature of the cystic fibrosis antigen. *Nature* **331**: 570.

Burges, J., Vincent, A., Molenaar, P. C., Newsom-Davis, J., Peers, C. and Wray, D. 1994. Passive transfer of seronegative myasthenia gravis to mice. *Muscle Nerve* **17**: 1393-400.

Burmeister, G., Tarcsay, L. and Sorg, C. 1986. Generation and characterization of a monoclonal antibody (1C5) to human migration inhibitory factor (MIF). *Immunobiology* **171**: 461-74.

Carvalho, T., Seeler, J. S., Ohman, K., Jordan, P., Pettersson, U., Akusjarvi, G., Carmo-Fonseca, M. and Dejean, A. 1995. Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. *J Cell Biol* **131**: 45-56.

Chakravarti, D., Pelling, J. C., Cavalieri, E. L. and Rogan, E. G. 1995. Relating aromatic hydrocarbon-induced DNA adducts and c-H-ras mutations in mouse skin papillomas: the role of apurinic sites. *Proc Natl Acad Sci U S A* **92**: 10422-6.

Chanarin, I. 1989. *Laboratory haematology : an account of laboratory techniques*. New York: Churchill Livingstone. 450 pp.

Chang, K. S., Fan, Y. H., Andreeff, M., Liu, J. and Mu, Z. M. 1995. The PML gene encodes a phosphoprotein associated with the nuclear matrix. *Blood* **85**: 3646-53.

Chen, G. Q., Zhu, J., Shi, X. G., Ni, J. H., Zhong, H. J., Si, G. Y., Jin, X. L., Tang, W., Li, X. S., Xong, S. M., Shen, Z. X., Sun, G. L., Ma, J., Zhang, P.,

Zhang, T. D., Gazin, C., Naoe, T., Chen, S. J., Wang, Z. Y. and Chen, Z. 1996. In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. *Blood* **88**: 1052-61.

Chen, S. J., Zelent, A., Tong, J. H., Yu, H. Q., Wang, Z. Y., Derre, J., Berger, R., Waxman, S. and Chen, Z. 1993a. Rearrangements of the retinoic acid receptor alpha and promyelocytic leukemia zinc finger genes resulting from t(11;17)(q23;q21) in a patient with acute promyelocytic leukemia. *J Clin Invest* **91**: 2260-7.

Chen, Z., Brand, N. J., Chen, A., Chen, S. J., Tong, J. H., Wang, Z. Y., Waxman, S. and Zelent, A. 1993b. Fusion between a novel Kruppel-like zinc finger gene and the retinoic acid receptor-alpha locus due to a variant t(11;17) translocation associated with acute promyelocytic leukaemia. *Embo J* **12**: 1161-7.

Chytil, F. 1996. Retinoids in lung development. *Faseb J* **10**: 986-92.

Clark, J. H., Hardin, J. W., Padykula, H. A. and Cardasis, C. A. 1978. Role of estrogen receptor binding and transcriptional activity in the stimulation of hyperestrogenism and nuclear bodies. *Proc Natl Acad Sci U S A* **75**: 2781-4.

Coligan, J., Ed. 1991. *Current protocols in immunology*. New York: Greene Publishing Associates and Wiley Interscience. pp. 1301-1301 pp.

Cordonnier, C., Vernant, J. P., Brun, B., Heilmann, M. G., Kuentz, M., Bierling, P., Farcet, J. P., Rodet, M., Duedari, N., Imbert, M. and et al. 1985. Acute promyelocytic leukemia in 57 previously untreated patients. *Cancer* **55**: 18-25.

Corey, S. J., Locker, J., Oliveri, D. R., Shekhter-Levin, S., Redner, R. L., Penchansky, L. and Gollin, S. M. 1994. A non-classical translocation

involving 17q12 (retinoic acid receptor alpha) in acute promyelocytic leukemia (APML) with atypical features. *Leukemia* **8**: 1350-3.

Cotsarelis, G., Sun, T. T. and Lavker, R. M. 1990. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* **61**: 1329-37.

Dale, I., Fagerhol, M. K. and Naesgaard, I. 1983. Purification and partial characterization of a highly immunogenic human leukocyte protein, the L1 antigen. *Eur J Biochem* **134**: 1-6.

Dalton, W. T., Jr., Ahearn, M. J., McCredie, K. B., Freireich, E. J., Stass, S. A. and Trujillo, J. M. 1988. HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood* **71**: 242-7.

Daniel, M. T., Koken, M., Romagne, O., Barbey, S., Bazarbachi, A., Stadler, M., Guillemin, M. C., Degos, L., Chomienne, C. and de The, H. 1993. PML protein expression in hematopoietic and acute promyelocytic leukemia cells. *Blood* **82**: 1858-67.

Darnell, R. B. 1996. Onconeural antigens and the paraneoplastic neurologic disorders: At the intersection of cancer, immunity, and the brain. *Proc Natl Acad Sci U S A* **93**: 4529-36.

Davey, F. R., Davis, R. B., MacCallum, J. M., Nelson, D. A., Mayer, R. J., Ball, E. D., Griffin, J. D., Schiffer, C. A. and Bloomfield, C. D. 1989. Morphologic and cytochemical characteristics of acute promyelocytic leukemia. *Am J Hematol* **30**: 221-7.

de The, H., Chomienne, C., Lanotte, M., Degos, L. and Dejean, A. 1990. The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus. *Nature* **347**: 558-61.

de The, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L. and Dejean, A. 1991. The PML-RAR alpha fusion mRNA generated by the t(15;17)

translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell* **66**: 675-84.

Degos, L. 1994. Is acute promyelocytic leukemia a curable disease? Treatment strategy for a long-term survival. *Leukemia* **8**: 911-3.

Desbois, C., Rousset, R., Bantignies, F. and Jalinot, P. 1996. Exclusion of Int-6 from PML nuclear bodies by binding to the HTLV-I Tax oncoprotein. *Science* **273**: 951-953.

Dolle, P., Ruberte, E., Leroy, P., Morriss-Kay, G. and Chambon, P. 1990. Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* **110**: 1133-51.

Dorin, J. R., Novak, M., Hill, R. E., Brock, D. J., Secher, D. S. and van Heyningen, V. 1987. A clue to the basic defect in cystic fibrosis from cloning the CF antigen gene. *Nature* **326**: 614-7.

Doucas, V., Brockes, J. P., Yaniv, M., de The, H. and Dejean, A. 1993. The PML-retinoic acid receptor alpha translocation converts the receptor from an inhibitor to a retinoic acid-dependent activator of transcription factor AP-1. *Proc Natl Acad Sci U S A* **90**: 9345-9.

Dyck, J. A., Maul, G. G., Miller, W. H., Jr., Chen, J. D., Kakizuka, A. and Evans, R. M. 1994. A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell* **76**: 333-43.

Early, E., Moore, M. A. S., Kakizuka, A., Nason-Burchenal, K., Martin, P., Evans, R. M. and Dmitrovsky, E. 1996. Transgenic expression of PML/RARα impairs myeloipoiesis. *Proc. Natl. Acad. Sci. USA* **93**: 7900-7904.

Everett, R. D. and Maul, G. G. 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. *Embo J* **13**: 5062-9.

Fanjul, M., Renaud, W., Merten, M., Guy-Crotte, O., Hollande, E. and Figarella, C. 1995. Presence of MRP8 and MRP14 in pancreatic cell lines: differential expression and localization in CFPAC-1 cells. *Am J Physiol* **268**: C1241-51.

Fenaux, P. and Chomienne, C. 1996. Biology and treatment of acute promyelocytic leukemia. *Curr. Opin. Oncol.* **8**: 3-12.

Fitzgerald, M. and Padykula, H. A. 1983. Differing functional responses of simple and complex nuclear bodies in uterine luminal epithelial cells following estrogenic stimuli. *Anat Rec* **205**: 131-41.

Fleming, T. J., Fleming, M. L. and Malek, T. R. 1993. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol* **151**: 2399-408.

Frankel, S. R., Eardley, A., Lauwers, G., Weiss, M. and Warrell, R. P., Jr. 1992. The "retinoic acid syndrome" in acute promyelocytic leukemia. *Ann Intern Med* **117**: 292-6.

Frazier, C. N. and Hu, C. u.-K. u. 1931. Cutaneous lesions associated with a deficiency in vitamin A in man. *Arch Int Med* **48**: 507-514.

Freemont, P. S., Hanson, I. M. and Trowsdale, J. 1991. A novel cysteine-rich sequence motif. *Cell* **64**: 483-4.

Fuchs, E. and Byrne, C. 1994. The epidermis: rising to the surface. *Curr Opin Genet Dev* **4**: 725-36.

Fukutani, H., Naoe, T., Ohno, R., Yoshida, H., Miyawaki, S., Shimazaki, C., Miyake, T., Nakayama, Y., Kobayashi, H., Goto, S., Takeshita, A., Kobayashi, S., Kato, Y., Shiraishi, K., Sasada, M., Ohtake, S., Murakami, H., Kobayashi, M., Endo, N., Shindo, H., Matsushita, K., Hasegawa, S., Tsuji, K., Ueda, Y., Tominaga, N., Furuya, H., Inoue, Y., Takeuchi, J., Morishita, H., Iida,

H. and al., e. 1995. Isoforms of PML-retinoic acid receptor alpha fused transcripts affect neither clinical features of acute promyelocytic leukemia nor prognosis after treatment with all-trans retinoic acid. The Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). *Leukemia* **9**: 1478-82.

Gabrielsen, T. O., Dale, I., Brandtzaeg, P., Hoel, P. S., Fagerhol, M. K., Larsen, T. E. and Thune, P. O. 1986. Epidermal and dermal distribution of a myelomonocytic antigen (L1) shared by epithelial cells in various inflammatory skin diseases. *J Am Acad Dermatol* **15**: 173-9.

Gaub, M. P., Rochette-Egly, C., Lutz, Y., Ali, S., Matthes, H., Scheuer, I. and Chambon, P. 1992. Immunodetection of multiple species of retinoic acid receptor alpha: evidence for phosphorylation. *Exp Cell Res* **201**: 335-46.

Gilbert, R. D., Karabus, C. D. and Mills, A. E. 1987. Acute promyelocytic leukemia. A childhood cluster. *Cancer* **59**: 933-5.

Goddard, A. D., Borrow, J., Freemont, P. S. and Solomon, E. 1991. Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia. *Science* **254**: 1371-4.

Goddard, A. D., Borrow, J. and Solomon, E. 1992. A previously uncharacterized gene, PML, is fused to the retinoic acid receptor alpha gene in acute promyelocytic leukaemia. *Leukemia* **6**: 117S-119S.

Green, E. L. 1966. *Biology of the laboratory mouse.* New York: Blakiston Division, McGraw-Hill. 2d ed. 706 pp.

Grignani, F., Fagioli, M., Alcalay, M., Longo, L., Pandolfi, P. P., Donti, E., Biondi, A., Lo Coco, F., Grignani, F. and Pelicci, P. G. 1994. Acute promyelocytic leukemia: from genetics to treatment. *Blood* **83**: 10-25.

Grignani, F., Ferrucci, P. F., Testa, U., Talamo, G., Fagioli, M., Alcalay, M., Mencarelli, A., Grignani, F., Peschle, C., Nicoletti, I. and Pelicci, P. G. 1993.

The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. *Cell* **74**: 423-31.

Grignani, F., Testa, U., Fagioli, M., Barberi, T., Masciulli, R., Mariani, G., Peschle, C. and Pelicci, P. G. 1995. Promyelocytic leukemia-specific PML-retinoic acid alpha receptor fusion protein interferes with erythroid differentiation of human erythroleukemia K562 cells. *Cancer Res* **55**: 440-3.

Grignani, F., Testa, U., Rogaia, R., Ferruci, P. F., Samoggia, P., Pinto, A., Aldinucci, D., Gelmetti, V., Fagioli, M., Alcalay, M., Seeler, J., Grignani, F., Nicoletti, I., Peschle, C. and Pelicci, P. G. 1996. Effects on differentiation by the promyelocytic leukemia PML/RARα protein depend on the fusion of the PML protein dimerization and RARα DNA binding domain. *EMBO Journal* **15**: 4949-4958.

Grisolano, J. L., Sclar, G. M. and Ley, T. J. 1994. Early myeloid cell-specific expression of the human cathepsin G gene in transgenic mice. *Proc Natl Acad Sci U S A* **91**: 8989-93.

Grisolano, J. L., Wesselschmidt, R. L., Pelicci, P. G. and Ley, T. L. Altered myeloid development and acute leukemia in transgenic mice expressing PML-RARα under control of cathepsin G regulatory sequences. *Blood* **In press**:

Guiochon-Mantel, A., Savouret, J. F., Quignon, F., Delabre, K., Milgrom, E. and De The, H. 1995. Effect of PML and PML-RAR on the transactivation properties and subcellular distribution of steroid hormone receptors. *Mol Endocrinol* **9**: 1791-803.

Hayward, C., Chung, S., Brock, D. J. and Van Heyningen, V. 1986. Monoclonal antibodies to cystic fibrosis antigen. *J Immunol Methods* **91**: 117-22.

Hennings, H., Glick, A. B., Lowry, D. T., Krsmanovic, L. S., Sly, L. M. and Yuspa, S. H. 1993. FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin. *Carcinogenesis* **14**: 2353-8.

Hestdal, K., Ruscetti, F. W., Ihle, J. N., Jacobsen, S. E., Dubois, C. M., Kopp, W. C., Longo, D. L. and Keller, J. R. 1991. Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *J Immunol* **147**: 22-8.

Hillestad, L. 1957. Acute promyelocytic leukemia. *Acta Medica Scand* **159**: 189-194.

Hodak, E., Jones, R. E. and Ackerman, A. B. 1993. Solitary keratoacanthoma is a squamous-cell carcinoma: three examples with metastases. *Am J Dermatopathol* **15**: 332-42; discussion 343-52.

Hogan, B., Constantini, F. and Lacy, E. 1994. *Manipulating the mouse embryo: a laboratory manual.* Plainview, NY: Cold Spring Harbor Laboratory Press. 2nd. 497 pp.

Huang, M. E., Ye, Y. C., Chen, S. R., Zhao, J. C., Gu, L. J., Cai, J. R., Zhao, L., Xie, J. X., Shen, Z. X. and Wang, Z. Y. 1987a. All-trans retinoic acid with or without low dose cytosine arabinoside in acute promyelocytic leukemia. Report of 6 cases. *Chin Med J (Engl)* **100**: 949-53.

Huang, M. E., Ye, Y. C. and Zhao, L. 1987b. Treatment of acute promyelocytic leukemia by retinoic acid with or without low dose cytosine arabinoside: report of 4 cases. *Chung Hua Nei Ko Tsa Chih* **26**: 330-2, 380.

Imakado, S., Bickenbach, J. R., Bundman, D. S., Rothnagel, J. A., Attar, P. S., Wang, X. J., Walczak, V. R., Wisniewski, S., Pote, J., Gordon, J. S. and et al. 1995. Targeting expression of a dominant-negative retinoic acid receptor

mutant in the epidermis of transgenic mice results in loss of barrier function. *Genes Dev* **9**: 317-29.

Jansen, J. H., Mahfoudi, A., Rambaud, S., Lavau, C., Wahli, W. and Dejean, A. 1995. Multimeric complexes of the PML-retinoic acid receptor alpha fusion protein in acute promyelocytic leukemia cells and interference with retinoid and peroxisome-proliferator signaling pathways. *Proc Natl Acad Sci U S A* **92**: 7401-5.

Jarrett, A. 1980. *The Mucus membranes : the action of vitamin A on the skin and mucus membranes : transepidermal water loss.* New York: Academic Press. 1872-2155 pp.

Jiang, W. Q., Szekely, L., Wendel-Hansen, V., Ringertz, N., Klein, G. and Rosen, A. 1991. Co-localization of the retinoblastoma protein and the Epstein-Barr virus-encoded nuclear antigen EBNA-5. *Exp Cell Res* **197**: 314-8.

Kakizuka, A., Miller, W., Jr., Umesono, K., Warrell, R., Jr., Frankel, S. R., Murty, V. V., Dmitrovsky, E. and Evans, R. M. 1991. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* **66**: 663-74.

Kastner, P., Perez, A., Lutz, Y., Rochette, E. C., Gaub, M. P., Durand, B., Lanotte, M., Berger, R. and Chambon, P. 1992. Structure, localization and transcriptional properties of two classes of retinoic acid receptor alpha fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncoproteins. *Embo J* **11**: 629-42.

Kerschmann, R. L., McCalmont, T. H. and LeBoit, P. E. 1994. p53 oncoprotein expression and proliferation index in keratoacanthoma and squamous cell carcinoma. *Arch Dermatol* **130**: 181-6.

King, A. 1853. A case of chloroma. *Monthly J. Medicine* **17**: 97.

Knowles, D. M. 1992. *Neoplastic hematopathology*. Baltimore: Williams & Wilkins. 1623 pp.

Koeffler, H. P. and Amatruda, T. T. d. 1985. The effect of retinoids on haemopoiesis--clinical and laboratory studies. *Ciba Found Symp* **113**: 252-67.

Koken, M., Reid, A., Quignon, F., Dong, S., Chen, Z., Waxman, S., de The, H. and Zelent, A. 1996. Products of the *PML* and *PLZF* genes translocated with the *RARα* locus in acute promyelocytic leukemia co-localize in the nucleus and interact with each other *in vivo*. *Blood* **88 Suppl. 1, Pt. 1**: 553a.

Koken, M. H., Linares-Cruz, G., Quignon, F., Viron, A., Chelbi-Alix, M. K., Sobczak-Thepot, J., Juhlin, L., Degos, L., Calvo, F. and de The, H. 1995. The PML growth-suppressor has an altered expression in human oncogenesis. *Oncogene* **10**: 1315-24.

Koken, M. H., Puvion-Dutilleul, F., Guillemin, M. C., Viron, A., Linares-Cruz, G., Stuurman, N., de Jong, L., Szostecki, C., Calvo, F., Chomienne, C., Degos, L., Puvion, E. and de The, H. 1994. The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. *Embo J* **13**: 1073-83.

Korioth, F., Gieffers, C., Maul, G. G. and Frey, J. 1995. Molecular characterization of NDP52, a novel protein of the nuclear domain 10, which is redistributed upon virus infection and interferon treatment. *J Cell Biol* **130**: 1-13.

Labelle, Y., Zucman, J., Stenman, G., Kindblom, L. G., Knight, J., Turc-Carel, C., Dockhorn-Dworniczak, B., Mandahl, N., Desmaze, C., Peter, M., Aurias, A., Delattre, O. and Thomas, G. 1995. Oncogenic conversion of a novel orphan nuclear receptor by chromosome translocation. *Hum Mol Genet* **4**: 2219-26.

Lagasse, E. and Weissman, I. L. 1994. bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages. *J Exp Med* **179**: 1047-52.

Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R. and Berns, A. 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res* **19**: 4293.

Lanotte, M., Martin-Thouvenin, V., Najman, S., Balerini, P., Valensi, F. and Berger, R. 1991. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood* **77**: 1080-6.

Lanzi, C., Borrello, M. G., Bongarzone, I., Migliazza, A., Fusco, A., Grieco, M., Santoro, M., Gambetta, R. A., Zunino, F., Della Porta, G. and Pierotti, M. A. 1992. Identification of the product of two oncogenic rearranged forms of the RET proto-oncogene in papillary thyroid carcinomas. *Oncogene* **7**: 2189-94.

Larson, R. A., Kondo, K., Vardiman, J. W., Butler, A. E., Golomb, H. M. and Rowley, J. D. 1984. Evidence for a 15;17 translocation in every patient with acute promyelocytic leukemia. *Am J Med* **76**: 827-41.

Licht, J. D., Chomienne, C., Goy, A., Chen, A., Scott, A. A., Head, D. R., Michaux, J. L., Wu, Y., DeBlasio, A., Miller, W. H., Jr., Zelenetz, A. D., Willman, C. L., Chen, Z., Chen, S.-J., Zelent, A., Macintyre, E., Veil, A., Cortes, J., Kantarjian, H. and Waxman, S. 1995. Clinical and molecular characterization of a rare syndrome of acute promyelocytic leukemia associated with translocation (11;17). *Blood* **85**: 1083-94.

Liu, J. H., Mu, Z. M. and Chang, K. S. 1995. PML suppresses oncogenic transformation of NIH/3T3 cells by activated neu. *J Exp Med* **181**: 1965-73.

Lo Coco, F., Diverio, D., D'Adamo, F., Avvisati, G., Alimena, G., Nanni, M., Alcalay, M., Pandolfi, P. P. and Pelicci, P. G. 1992. PML/RAR-alpha rearrangement in acute promyelocytic leukaemias apparently lacking the t(15;17) translocation. *Eur J Haematol* **48**: 173-6.

Lohnes, D., Mark, M., Mendelsohn, C., Dolle, P., Dierich, A., Gorry, P., Gansmuller, A. and Chambon, P. 1994. Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development* **120**: 2723-48.

Lugering, N., Stoll, R., Kucharzik, T., Schmid, K. W., Rohlmann, G., Burmeister, G., Sorg, C. and Domschke, W. 1995. Immunohistochemical distribution and serum levels of the Ca(2+)-binding proteins MRP8, MRP14 and their heterodimeric form MRP8/14 in Crohn's disease. *Digestion* **56**: 406-14.

Mader, S., Chen, J. Y., Chen, Z., White, J., Chambon, P. and Gronemeyer, H. 1993. The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificites of the DNA binding domains. *Embo J* **12**: 5029-41.

Maeda, Y., Horiuchi, F., Miyatake, J., Sono, H., Tatsumi, Y., Urase, F., Irimajiri, K. and Horiuchi, A. 1996. Inhibition of growth and induction of apoptosis by all-trans retinoic acid in lymphoid cell lines transfected with the PML/RAR alpha fusion gene. *Br J Haematol* **93**: 973-6.

Mangelsdorf, D. J. and Evans, R. M. 1995a. The RXR heterodimers and orphan receptors. *Cell* **83**: 841-50.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. M. 1995b. The nuclear receptor superfamily: the second decade. *Cell* **83**: 835-9.

Marshall, H., Morrison, A., Studer, M., Popperl, H. and Krumlauf, R. 1996. Retinoids and Hox genes. *Faseb J* **10**: 969-78.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P. and Mark, M. 1994. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120**: 2749-71.

Miki, T., Fleming, T. P., Crescenzi, M., Molloy, C. J., Blam, S. B., Reynolds, S. H. and Aaronson, S. A. 1991. Development of a highly efficient expression cDNA cloning system: application to oncogene isolation. *Proc Natl Acad Sci U S A* **88**: 5167-71.

Morosetti, R., Grignani, F., Liberatore, C., Pelicci, P. G., Schiller, G. J., Kizaki, M., Bartram, C. R., Miller, C. W. and Koeffler, H. P. 1996. Infrequent alterations of the RAR alpha gene in acute myelogenous leukemias, retinoic acid-resistant acute promyelocytic leukemias, myelodysplastic syndromes, and cell lines. *Blood* **87**: 4399-403.

Mu, Z. M., Chin, K. V., Liu, J. H., Lozano, G. and Chang, K. S. 1994. PML, a growth suppressor disrupted in acute promyelocytic leukemia. *Mol Cell Biol* **14**: 6858-67.

Muindi, J. R., Young, C. W. and Warrell, R. P., Jr. 1994. Clinical pharmacology of all-trans retinoic acid. *Leukemia* **8 Suppl 3**: S16-21.

Najfeld, V., Scalise, A. and Troy, K. 1989. A new variant translocation 11;17 in a patient with acute promyelocytic leukemia together with t(7;12). *Cancer Genet Cytogenet* **43**: 103-8.

Nakjang, Y. and Yuttanavivat, T. 1988. Phrynoderma: a review of 105 cases. *J Dermatol* **15**: 531-4.

Nason-Burchenal, K., Gandini, D., Botto, M., Allopenna, J., Seale, J., Cross, N., Goldman, J., Dmitrovsky, E. and Pandolfi, P. 1996. Interferon augments

PML and PML/RARα expression in normal myeloid and acute promyelocytic leukemia cells and cooperates with all-*trans* retinoic acid to induce maturation of a retinoid-resistant promyelocytic cell line. *Blood* **88**: 3926-3936.

Odink, K., Cerletti, N., Bruggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R. and Sorg, C. 1987. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature* **330**: 80-2.

Ojeda, V. J. 1984. Necrotizing myelopathy associated with malignancy. A clinicopathologic study of two cases and literature review. *Cancer* **53**: 1115-23.

Onodera, M. 1994. An overexpression of retinoic acid receptor alpha blocks myeloid cell differentiation at the promyelocyte stage. *Hokkaido Igaku Zasshi* **69**: 466-75, 477.

Onodera, M., Kunisada, T., Nishikawa, S., Sakiyama, Y., Matsumoto, S. and Nishikawa, S. 1995. Overexpression of retinoic acid receptor alpha suppresses myeloid cell differentiation at the promyelocyte stage. *Oncogene* **11**: 1291-8.

Paggi, M. G., de Fabritiis, P., Bonetto, F., Amadio, L., Santarelli, G., Spadea, A., Gentile, F. P., Floridi, A. and Felsani, A. 1995. The retinoblastoma gene product in acute myeloid leukemia: a possible involvement in promyelocytic leukemia. *Cancer Res* **55**: 4552-6.

Paietta, E., Andersen, J., Gallagher, R., Bennett, J., Yunis, J., Cassileth, P., Rowe, J. and Wiernik, P. H. 1994. The immunophenotype of acute promyelocytic leukemia (APL): an ECOG study. *Leukemia* **8**: 1108-12.

Pandolfi, P. P., Alcalay, M., Fagioli, M., Zangrilli, D., Mencarelli, A., Diverio, D., Biondi, A., Lo Coco, F., Rambaldi, A. and Grignani, F. 1992. Genomic variability and alternative splicing generate multiple PML/RAR

alpha transcripts that encode aberrant PML proteins and PML/RAR alpha isoforms in acute promyelocytic leukaemia. *Embo J* **11**: 1397-407.

Pandolfi, P. P., Grignani, F., Alcalay, M., Mencarelli, A., Biondi, A., LoCoco, F., Grignani, F. and Pelicci, P. G. 1991. Structure and origin of the acute promyelocytic leukemia myl/RAR alpha cDNA and characterization of its retinoid-binding and transactivation properties. *Oncogene* **6**: 1285-92.

Perez, A., Kastner, P., Sethi, S., Lutz, Y., Reibel, C. and Chambon, P. 1993. PMLRAR homodimers: distinct DNA binding properties and heteromeric interactions with RXR. *Embo J* **12**: 3171-82.

Pinkus, H. and Mehregan, A. H. 1981. *A guide to dermatohistopathology.* New York: Appleton-Century-Crofts. 3d ed. 591 pp.

Raelson, J. V., Nervi, C., Rosenauer, A., Benedetti, L., Monczak, Y., Pearson, M., Pelicci, P. G. and Miller, W. H. 1996. The PML/RARα oncoprotein is a direct molecular target of retinoic acid in acute promyelocytic leukemia cells. *Blood* **88**: 2826-2832.

Reddy, B. A. and Etkin, L. D. 1991. A unique bipartite cysteine-histidine motif defines a subfamily of potential zinc-finger proteins. *Nucleic Acids Res* **19**: 6330.

Reddy, B. A., Etkin, L. D. and Freemont, P. S. 1992. A novel zinc finger coiled-coil domain in a family of nuclear proteins. *Trends Biochem Sci* **17**: 344-5.

Redner, R. L., Rush, E. A., Faas, S., Rudert, W. A. and Corey, S. J. 1996. The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion. *Blood* **87**: 882-6.

Rivi, R., Calleja, E., Konig, A., Lai, L., Gambacorti-Passerini, C., Scheinberg, D., Gabrilove, J., Warrell, R. J. and Pandolfi, P. P. 1996. Organic arsenical melarsoprol shows growth suppressive activity via programmed

cell death on APL and other myeloid and lymphoid leukemia derived cell lines. *Blood* **88 Suppl. 1, Pt. 1**: 68a.

Robbins, S. M., Quintrell, N. A. and Bishop, J. M. 1995. Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachment to membranes and association with caveolae. *Mol Cell Biol* **15**: 3507-15.

Rogaia, D., Grignani, F., Grignani, F., Nicoletti, I. and Pelicci, P. G. 1995. The acute promyelocytic leukemia-specific PML/RAR alpha fusion protein reduces the frequency of commitment to apoptosis upon growth factor deprivation of GM-CSF-dependent myeloid cells. *Leukemia* **9**: 1467-72.

Rook, A., Burton, J. L., Champion, R. H., Ebling, F. J. G. and Wilkinson, D. S. 1992. *Textbook of dermatology*. Oxford: Blackwell Scientific. 5th ed. 3160 pp.

Rousselot, P., Hardas, B., Patel, A., Guidez, F., Gaken, J., Castaigne, S., Dejean, A., de The, H., Degos, L., Farzaneh, F. and Chomienne, C. 1994. The PML-RAR alpha gene product of the t(15;17) translocation inhibits retinoic acid-induced granulocytic differentiation and mediated transactivation in human myeloid cells. *Oncogene* **9**: 545-51.

Rowley, J. D., Golomb, H. M. and Dougherty, C. 1977. 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia. *Lancet* **1**: 549-50.

Saitou, M., Sugai, S., Tanaka, T., Shimouchi, K., Fuchs, E., Narumiya, S. and Kakizuka, A. 1995. Inhibition of skin development by targeted expression of a dominant-negative retinoic acid receptor. *Nature* **374**: 159-62.

Schuh, A. C., Keating, S. J., Monteclaro, F. S., Vogt, P. K. and Breitman, M. L. 1990. Obligatory wounding requirement for tumorigenesis in v-jun transgenic mice. *Nature* **346**: 756-60.

Sommer, A. and West, K. P. 1996. *Vitamin A deficiency : health, survival, and vision*. New York: Oxford University Press. 438 pp.

Stolte, M. 1993. Histomorphological age changes and ultrastructural characteristics of the preputial and clitoral glands of mice. *J Exp Anim Sci* **35**: 166-76.

Stone, R. M. and Mayer, R. J. 1990. The unique aspects of acute promyelocytic leukemia. *J Clin Oncol* **8**: 1913-21.

Stuurman, N., de Graaf, A., Floore, A., Josso, A., Humbel, B., de Jong, L. and van Driel, R. 1992. A monoclonal antibody recognizing nuclear matrix-associated nuclear bodies. *J Cell Sci* **101**: 773-84.

Szekely, L., Pokrovskaja, K., Jiang, W. Q., de The, H., Ringertz, N. and Klein, G. 1996. The Epstein-Barr virus-encoded nuclear antigen EBNA-5 accumulates in PML-containing bodies. *J Virol* **70**: 2562-8.

Taketo, M., Schroeder, A. C., Mobraaten, L. E., Gunning, K. B., Hanten, G., Fox, R. R., Roderick, T. H., Stewart, C. L., Lilly, F., Hansen, C. T. and Overbeek, P. A. 1991. FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc Natl Acad Sci U S A* **88**: 2065-9.

Tang, T. K., Hong, T. M., Lin, C. Y., Lai, M. L., Liu, C. H., Lo, H. J., Wang, M. E., Chen, L. B., Chen, W. T., Ip, W. and et al. 1993. Nuclear proteins of the bovine esophageal epithelium. I. Monoclonal antibody W2 specifically reacts with condensed nuclei of differentiated superficial cells. *J Cell Sci* **104**: 237-47.

Terris, B., Baldin, V., Dubois, S., Degott, C., Flejou, J. F., Henin, D. and Dejean, A. 1995. PML nuclear bodies are general targets for inflammation and cell proliferation. *Cancer Res* **55**: 1590-7.

Tsai, S., Bartelmez, S., Heyman, R., Damm, K., Evans, R. and Collins, S. J. 1992. A mutated retinoic acid receptor-α exhibiting dominant-negative

activity alters the lineage development of a multipotent hematopoietic cell line. *Genes Dev.* **6**: 2258-2269.

Tsai, S., Bartelmez, S., Sitnicka, E. and Collins, S. 1994. Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development. *Genes Dev* **8**: 2831-41.

Tsai, S. and Collins, S. J. 1993. A dominant negative retinoic acid receptor blocks neutorphil development at the promyelocyte stage. *PNAS* **90**: 7153-7157.

Vahlquist, A., Andersson, E., Coble, B. I., Rollman, O. and Torma, H. 1996. Increased concentrations of 3,4-didehydroretinol and retinoic acid-binding protein (CRABPII) in human squamous cell carcinoma and keratoacanthoma but not in basal cell carcinoma of the skin. *J Invest Dermatol* **106**: 1070-4.

Wang, Z. G., Giorgio, M., Rivi, R., Cordon-Cardo, C., Gaboli, M. and Pandolfi, P. P. 1996. Growth and tumor suppressive properties of the *PML* gene of acute promyelocytic leukemia in *PML* -/- mice. *Blood* **88 Suppl. 1, Pt. 1**: 476a.

Warrell, R. J., Frankel, S. R., Miller, W. J., Scheinberg, D. A., Itri, L. M., Hittelman, W. N., Vyas, R., Andreeff, M., Tafuri, A. and Jakubowski, A. 1991. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). *N Engl J Med* **324**: 1385-93.

Warrell, R. P., Jr., de The, H., Wang, Z. Y. and Degos, L. 1993. Acute promyelocytic leukemia. *N Engl J Med* **329**: 177-89.

Wechsler, H. L. 1979. Vitamin A deficiency following small-bowel bypass surgery for obesity. *Arch Dermatol* **115**: 73-5.

Weinberg, R. A. 1995. The retinoblastoma protein and cell cycle control. *Cell* **81**: 323-30.

Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A. and Dejean, A. 1994. Retinoic acid regulates aberrant nuclear localization of PML-RAR alpha in acute promyelocytic leukemia cells. *Cell* **76**: 345-56.

Wilkinson, M. M., Busuttil, A., Hayward, C., Brock, D. J., Dorin, J. R. and Van Heyningen, V. 1988. Expression pattern of two related cystic fibrosis-associated calcium-binding proteins in normal and abnormal tissues. *J Cell Sci* **91**: 221-30.

Wistow, G. J., Shaughnessy, M. P., Lee, D. C., Hodin, J. and Zelenka, P. S. 1993. A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens. *Proc Natl Acad Sci U S A* **90**: 1272-5.

Wolbach, S. B. and Howe, P. R. 1925. Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.* **30**: 753-777.

Xie, K., Lambie, E. J. and Snyder, M. 1993. Nuclear dot antigens may specify transcriptional domains in the nucleus. *Mol Cell Biol* **13**: 6170-9.

Yoshida, H., Kitamura, K., Tanaka, K., Omura, S., Miyazaki, T., Hachiya, T., Ohno, R. and Naoe, T. 1996. Accelerated degradation of PML-retinoic acid receptor alpha (PML-RARA) oncoprotein by all-trans-retinoic acid in acute promyelocytic leukemia: possible role of the proteasome pathway. *Cancer Res* **56**: 2945-8.

Zwadlo, G., Bruggen, J., Gerhards, G., Schlegel, R. and Sorg, C. 1988. Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clin Exp Immunol* **72**: 510-5.