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BIOLOGICAL FUNCTIONS OF PROMYELOCYTIC LEUKEMIA ZINC FINGER PROTEIN (PLZF)

by

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

BIOLOGICAL FUNCTIONS OF PROMYELOCYTIC LEUKEMIA ZINC FINGER PROTEIN (PLZF)

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Advisor: Jonathan D. Licht, M.D.

The unusual balanced chromosomal translocation t(11;17) (q23;q21) is associated with acute promyelocytic leukemia which unlike the more typical t(15;17) APL, fails to respond to treatment with all-trans retinoic acid. In t(11;17) the PLZF gene on chromosome 11 is fused to the retinoic acid receptor α gene on chromosome 17 and yields two reciprocal fusion proteins. Using anti-PLZF antibodies, I characterized the nature of the wild-type PLZF protein and its fusion proteins with the retinoic acid receptor. PLZF gene yields a 90 kd nuclear protein. The PLZF-RARα fusion protein is also localized exclusively to the nucleus, while the reciprocal RARα-PLZF protein is found in both the nucleus and cytoplasm. I also demonstrated that PLZF is phosphorylated; upon further examination it was determined that PLZF is phosphorylated on serine and threonine residues. I investigated the effect of PLZF on differentiation of the nontumorigenic mouse myeloid cell line 32DCL3G/GM. Overexpression of PLZF had a dramatic growth suppressive effect on these cells. Severe growth

suppression was accompanied by accumulation of cells in the G0/G1 compartment of the cell cycle, an increased incidence of apoptosis and downregulation of IL-3 receptor expression. There is also evidence of cell-autonomous mechanism of growth suppression through a putative autocrine factor secreted by pools of PLZF expressing cells, which inhibits growth of cells not expressing PLZF. PLZF overexpression inhibits the granulocytic and monocytic differentiation of 32DCL3G/GM cells in response to G-CSF and GM-CSF respectively. Furthermore, cells that express PLZF become more immature as demonstrated by their morphology-- increased expression of Sca-1 and decreased expression of Gr-1. These findings suggest that PLZF plays an important role in control of the cell cycle, cell death and cell differentiation. Disruption of PLZF in t(11;17) may be a critical event leading to hematopoietic malignancy.

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Chapter I

Introduction

1. Normal hematopoiesis: from stem cells to peripheral blood.

Blood is a multiorgan liquid tissue, which consists of plasma (containing numerous proteins and minerals) and a suspension of white cells (leukocytes), plasma cells (thrombocytes) and red cells (erythrocytes) (Simmons, 1997). Embryonic hematopoiesis starts in the yolk sac from mesenchymal cells of the embryoid connective tissue that form blood islands and signifies the mesoblastic stage. The yolk sac contains totipotent hematopoietic stem cells (THSC) that are capable of self-renewal and production of pluripotent stem cells or progenitors, which have undergone determination with the loss of self-renewing capacity but gained capacity to give rise to differentiated cells of various lineages (Coulombel et al., 1997; Dieterlen-Lievre et al., 1997). The cells on the outer layer of blood islands form a continuous vascular system, while the rest of blood island produces erythroidlike cells as early as 14 days. It has been recently demonstrated that aorta-gonad-mesemchyme region (AGM) is the site of the definitive pre-hepatic hematopoiesis (Medvinsky and Dzierzak, 1996).

The hepatic stage of hematopoiesis starts at around the third month of human embryonic development as the liver takes over the production of erythrocytes and granulocytes. At this stage, hematopoiesis also occurs in the spleen, thymus and the lymph nodes. Although the liver continues to produce blood until after birth, in the fourth month of embryonic development hematopoiesis within the bone marrow starts to play a more prominent role. In fact, bone marrow of the flat bones like sternum, ribs, skull and pelvis remains the main site of hematopoiesis throughout adult life (Simmons, 1997).

It is widely recognized that a small population of the totipotent hematopoietic stem cells (THSCs) give rise to multiple lineages forming a hierarchy of progressively more differentiated cells with documented molecular and morphological changes. THSCs give rise to a common progenitor for all hematopoietic lineages (Ogawa, 1993). This hypothesis is supported by experimental evidence which demonstrates a common origin for the myeloid and lymphoid lineages. Hirayama reported that preparation of bone marrow cells after 5-Fluorouracil (5-FU) treatment (eliminates actively dividing cells, but not more quiescent progenitors) contained progenitors with both myeloid and lymphoid potentials that could be expanded in a methylcellulose suspension culture system (Hirayama et al., 1992). During further commitment, lymphoid progenitors give rise to cells of T and B lymphoid lineages. Myeloid precursors give rise to at least four lineages: monocytic/granulocytic, erythrocytic, eosiniphilic and

megakaryocytic (Figure A). These committed cells undergo terminal differentiation, carry out their specialized functions and eventually senesce.

1A. The phenomenon of hematopoietic stem cells.

Totipotent stem cells are capable of self-renewal and can be identified by a functional assay. This assay, performed in mice, is the spleen colony-forming test (McCulloch, 1993). Hematopoietic cells are injected into lethally irradiated mouse and hematopoietic colonies in the spleen are counted several days later. The colonies are called Colony Forming Units - Spleen or CFU-S. The characteristics of the progenitor cells in terms of lineage potential and self-renewal capabilities can be inferred from the identity of cells that populate the splenic colonies, the order of their appearance and the longevity of the colony. The later appearing colonies arise from the more primitive cells and survive longer because of the greater self-renewing potential. After 6 days of transplantation into the irradiated mice small microscopic colonies appear that are erythroid in nature and survive for only 1-2 days. The transient nature and lineage restriction suggests that these progenitors were committed to differentiation and lost renewing capacity. By day 9-10 large colonies of mixed cellular content appear, but only transiently suggesting that they originate from pluripotent cells that can not self-renew. Finally by day 12-14, multilineage colonies can be seen that contain new colony-forming cells and are thought to originate from hematopoietic stem cells (Magli et al., 1982).

Hematopoietic stem cells constitute a very small subset of bone marrow cells, that is not easily identifiable at present. But despite their rarity, stem cells are capable of providing a life-long supply of hematopoietic cells of every lineage in the organism (Dexter et al., 1990; McCulloch et al., 1988; McCulloch et al., 1984; Necas, 1985). Hematopoietic stem cells are isolated from the adult mouse bone marrow based on cell surface characteristics and in some strains of mice they can be entirely contained in the population of cells containing low levels of T lymphocyte antigen Thy1.1, absence of markers for granulocytic, monocytic or erythroid lineages (Lin) and a high level of Sca-1 marker ($Thy1.1^{low}Lin^{neg}Sca^{+}$). Totipotent stem cells can be even further enriched based on their ability to uptake the vital mitochondrial dye rhodamine-123 (Rh-123). Half of the $Thy1.1^{low}Lin^{neg}Sca^{+}$ cells stain with Rh-123 and are highly enriched for short-term multipotent progenitors, but can not provide long-term repopulation. The other half of the cells with the low level of Rh-123 staining are capable of providing immediate radioprotection as well as life-long hematopoiesis in the transplanted animal (Spangrude, 1994; Spangrude et al., 1995), suggesting that totipotent stem cells can be even more precisely described as $Thy1.1^{low}Lin^{neg}Sca^{+}$ $Rh\text{-}123^{low}$ population. Transplantation of 100 $Thy1.1^{low}Lin^{neg}Sca^{+}$ cells protected 100% of irradiated animals, while transfer of 25 cells protected 35% of the animals. The implication of this result is that a critical number of the stem cells is necessary to reconstitute normal hematopoiesis and to protect an irradiated mouse. Only five genetically marked Rh-low cells added to 10^5 of normal bone marrow cells were sufficient to contribute 10% of cells to peripheral blood of irradiated animal, demonstrating the powerful proliferative and self-renewing potential of these cells. The fraction highly

enriched for stem cells constitutes approximately 0.05% of all bone marrow cells (Spangrude et al., 1995)

Stem cells are thought to be dormant in the cell cycle, residing in the G0 phase (Fleming et al., 1993; Lajtha, 1979). Short exposure of bone marrow cells to ^{3}H (tritiated thymidine) with high specific activity does not reduce the number of multipotential progenitors (Hara and Ogawa, 1978). In addition, treatment of donor mice with 5-fluorouracil (5-FU) does not inhibit appearence of day 12-14 spleen colonies (Suda et al., 1983). These two experiments demonstrate that stem cells are resistant to DNA damage suggesting that stem cells are quiescent and do not synthesize DNA. Leary *et al.* directly observed stem cells in culture and documented that these cells did not undergo cell division for as long as two weeks (Leary et al., 1989).

1B. Model of stem cell commitment and differentiation.

There are several models describing stem cell commitment. A stochastic model was proposed by Till *et al.* in 1964 (Till et al., 1964) and was based on the fact that stem cells in the spleen colony forming test gave rise to heterogeneous colonies during commitment with different ratios of macrophages, erythrocytes, neutrophils and basophils. Therefore, the

behavior of the new colony-forming cells can be fitted with gamma distribution, which describes a stochastic process with two options available at each cell division. For stem cell, each cell division leads to either self-renewal (birth of a new stem cell) or determination and the death of the stem cell. According to this model, birth and death occur at random, with balancing of these effects over time. This model also suggests that if self-renewal is not sufficiently frequent, stem cells would become extinct (McCulloch, 1993). In support of this model, it was demonstrated that multilineage colonies from a single cell produced a variety of lineage combinations. Two daughter cells, derived from a single cell give rise to different combinations of lineages (Suda et al., 1983). The stochastic model was based on the assumption that external factors do not influence the fate of the stem cells. In support of this assumption Mayani *et al.* took a fraction of human fetal cord blood that is enriched for multipotent progenitors and exposed these cells to different combinations of cytokines. They observed that the relative proportion of cells committed to different lineages remained the same, independently of the cytokine combination used (Mayani et al., 1993; Mayani et al., 1993).

A second, deterministic model of stem cell competition proposes that commitment versus self-renewal is determined under the influence of humoral factors like Interleukin-3 (IL-3) or Erythropoietin (Epo) (Van Zant and Goldwasser, 1979). Katayama *et al.* demonstrated that IL-3 and Steel factor are effective in supporting survival of stem cells, while IL-6, G-CSF and IL-11 encourage stem cells to exit the dormant stage and to proliferate. Metcalf reported that high concentrations of GM-CSF or GM-CSF and IL-3

in combination with the Steel factor support production of granulocytic progenitors (Metcalf, 1980), suggesting that extrinsic factors effect the fate of the stem cells.

It is possible that a model combining features of stochastic and deterministic models will reflect the real course of events in commitment of hematopoietic stem cells. Each experimental outcome is effected to various degrees by the design of the experiment resulting in conflicting pieces of evidence for the complex mechanism of commitment. This contradiction within itself suggests that at this point in time a pure fraction of stem cells can not be obtained and studied, and that instead the "stemness" as a wide spectrum of characteristics is under investigation.

1C. Role of transcription factors in hematopoiesis.

Throughout the development of hematopoietic cells transcription factors are critical in the execution of the developmental program. Many genes have been implicated in hematopoietic development based on the results of knock-out and knock-in mouse technology, genetic manipulations of ES cells with consequent study of *in vitro* differentiation, immunocytochemistry and *in situ* hybridization assays.

Little is known about transcriptional regulation of the hematopoietic progenitor cells, particularly during embryogenesis. Homeobox genes, that have been implicated as master morphogenic regulators during embryoid development, may contribute to the regulation of hematopoiesis. Genes from the A, B and C clusters of the homeobox genes are expressed in hematopoietic cell lines with distinct patterns. Genes located in the 3' side of the clusters are expressed in more pluripotent cells, while genes from the 5' side of the cluster have broader range and are downregulated later during lineage differentiation (Lawrence et al., 1996; Thorsteinsdottir et al., 1997). Among genes from the B cluster only HOX B3 is expressed in dormant progenitor cells, while HOX B4 and B5 are induced slightly later during determination and differentiation of erythroid and granulocytic lineages. B6 is exclusively expressed during granulocytic differentiation. Overexpression of the B4 gene in ES cells significantly increases the number of progenitor cells, suggesting its role in the proliferative potential of hematopoietic stem cells (Helgason et al., 1996). When antisense B3 oligos are added to purified progenitor cells they produce striking inhibition in the formation of erythroid and granulomonocytic colonies. Less pronounced inhibition of both classes of colonies is also observed with antisense B4 and B5 oligos (Giampaolo et al., 1994). On the other hand overexpression of A10 in murine bone marrow disrupts myeloid and B-lymphoid differentiation and causes acute myeloid leukemia when bone marrow cells are transplanted into mice (Thorsteinsdottir et al., 1997).

A complex and dynamic pattern of expression in blood lineages has also been observed for the members of the GATA family of transcription factors.

GATA proteins are zinc finger transcription factors that recognize a "GATA" DNA binding motif found in most of erythroid-specific genes as well as in the globin locus control regions (Evans et al., 1988; Orkin, 1990). GATA-2 is highly expressed in pluripotent progenitors; its expression in mast cells and megakaryocytes overlaps with GATA- 1, while GATA-3 is highly expressed in T-lymphoid cells (Leonard et al., 1993; Tsai et al., 1994; Weiss and Orkin, 1995). ES cells homozygous for GATA-1 disruption do not undergo erythroid differentiation, although they are capable of differentiating along other hematologic lineages (Weiss et al., 1994). When GATA-1 is reintroduced to the murine GATA-1-/- ES cells it rescues the phenotype, reinforcing the specificity of effect in erythroid compartment. Chicken GATA-1 was effective in the rescue experiments, demonstrating that GATA proteins are evolutionary conserved functionally and structurally and suggesting their vital importance for hematopoietic development (Blobel et al., 1995). Takahashi *et al.* mapped the enhancer in the promoter of GATA-1 gene to -3.9 - -2.6 kB 5' from the first exon. This element is sufficient to recapitulate GATA-1 expression in the primitive erythroid cells (Takahashi et al., 1997), supporting the specificity of expression and action of GATA-1 protein.

Myeloid lineage development is dependent on the transcription factor, Pu.1, which is a member of Ets family and a product of the *spi-1* gene. The *spi-1* gene is disrupted by insertional mutagenesis in Friend erythroleukemia, which is associated with upregulation of PU.1 expression (Delgado et al., 1994; Moreau-Gachelin, 1994; Moreau-Gachelin et al., 1990; Moreau-Gachelin et al., 1989; Scott et al., 1994). During

development PU.1 is expressed at low levels in ES cells and $CD34^+$ cells, but is upregulated during myeloid differentiation (Voso et al., 1993). PU.1 binds to the purine rich sequence GAGGAA, which is found in promoters of many myeloid genes including CD11b, G-CSF receptor, GM-CSF receptor and M-CSF receptor (Klemsz et al., 1990; Voso et al., 1993). Since PU.1 can bind TBP in vitro, it is thought to function by bringing the basal transcriptional machinery in proximity to myeloid promoters, which lack TATAA boxes (Hagemeier et al., 1993). It was observed that 50% of homozygous transgenic animals expressing PU.1 develop erythroleukemias within six months from birth, which parallels the *in vivo* observation of association of Friend erythroleukemia with significant PU.1 upregulation (Moreau-Gachelin et al., 1996). Mice that are homozygous null for PU.1 expression were generated by McKercher *et al.*. $PU.1^{-/-}$ animals were born, but had to be kept in sterile environment with administration of antibiotics in order to be kept alive, suggesting that they were severely immunocompromised (McKercher et al., 1996). These animals lacked monocytes and mature B cells, produced few and defective neutrophils , but did contain progenitor B cells. These experiments point to the existence of a common pathway in the development of mature myeloid and lymphoid B cells and to the important role of PU.1.

In addition to PU.1, C/EBPs (CCAAT/enhancer binding proteins) were implicated in myelocytic differentiation (Cao et al., 1991; Williams et al., 1991). C/EBP α, β, γ and ε proteins are members of the basic-leucine zipper family of transcription factors that homodimerize and heterodimerise with each other and contain a basic region that binds DNA (Landschulz et

al., 1988; McKnight et al., 1988). Mice homozygous for C/EBPα null mutation do not produce mature neutrophils (monocytic Mac-1 and granulocytic Gr-1 markers are significantly reduced). C/EBP-/- mice make normal T and B lymphocytes, suggesting that C/EBP is critical only for granulocytic differentiation (Flodby et al., 1996; Wang et al., 1995).

It is becoming clear that transcription factors form a complex network in hematopoietic lineages resulting in lineage differentiation. Some of the transcription factors like HOX B4 and GATA-2 provide proliferative potential to pluripotent cells. Others like c-myb and c-myc have to be downregulated in order for the differentiation program to proceed. Yet a third class is thought to be the master regulators, that are upregulated at the very beginning of lineage determination and govern the program to terminal differentiation, among them are GATA-1 for erythroid lineage, PU.1 for myeloid lineage and Ikaros for lymphoid lineage. In support of this hypothesis, the knockout of GATA-1 results in complete failure of erythroid cells to develop (Weiss et al., 1994), while the homozygous mutation in DNA binding domain of Ikaros results in arrest of lymphoid differentiation at an early stage (Georgopoulos et al., 1994; Georgopoulos et al., 1992; Molnar and Georgopoulos, 1994).

2. Hematologic malignancies.

The transformation of the normal cell into leukemic cell is usually a multistep process that is characterized by accumulation of genetic abnormalities. Genetic abnormalities include reciprocal translocations, inversions, deletions and point mutations in the genes that code for proteins that are intimately involved in cell proliferation, growth and differentiation. These critical proteins involve growth factors, growth factor receptors, members of signal transduction pathway and finally nuclear proto-oncogenes and tumor suppressor genes (Carter et al., 1992; Yunis and Tanzer, 1993).

The growth factor IL-3 is affected in t(5;14)(q31;q32.3) which is associated with pre-B cell ALL. As a result of the translocation, IL-3 expression falls under control of immunoglobulin heavy chain (IgH) enhancer, which leads to overexpression of IL-3 and an autoregulatory loop that contributes to leukemogenesis (Meeker et al., 1990). An example of an affected cell membrane growth factor receptor is a point mutation in the fms gene, which codes for colony stimulating factor 1 receptor (CSF 1 Receptor), and is associated with 10% of myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML) (Ridge et al., 1987). Binding of the CSF-1 to its receptor results in tyrosine phosphorylation of the receptor and the activation of phosphoinositol (PtdIns) kinase leading to signaling into the nucleus (Insogna et al., 1997; Stanley et al., 1997; Yen et al., 1996). Mutations at codons 301 and 969 are often found and can alter biological

properties of the receptor rendering it constitutively active, in the case of mutation 301 (Roussel et al., 1988; Roussel et al., 1988; Woolford et al., 1988). Point mutations in the ras genes (K, N or H ras) that lead to an activated signaling molecule, are found in various neoplasms. Point mutations in ras have been detected in 20% of AML (Ahuja et al., 1990; Ahuja et al., 1990; Bos et al., 1987; Lubbert et al., 1990; Neubauer et al., 1994), 40% of MDS (Paquette et al., 1993), in up to 50% of lung and colon cancers and in 95% of pancreatic carcinomas (Bos et al., 1987). Mutations occur at codons 12,13 and 61, that correspond to GTP-binding sites of ras and render RAS protein insensitive to GTPase-activating proteins p120 GAP and NF1, making RAS persist in the activated form as p21 GTP and to provide constitutive mitogenic signal that can transform cells (Hall, 1990; Heriche et al., 1997; Rodriguez-Viciana et al., 1997).

2A. Tumor suppressor gene commonly affected in hematologic malignancies.

Genetic defects in p53 are most commonly found in the human cancer (Levine et al., 1991). Mutations and deletions of p53 have been described in acute promyelocytic leukemia (APL), some lymphoid malignancies and in hematologic cell lines HL-60 and T-cell leukemia cell line. p53 is a 393 aa protein with a DNA binding domain that is inactivated by point mutations in the hot spot between amino acids 110 and 307 (el-Deiry et al., 1992). p53 can suppress phosphorylation of RB and can inhibit G1 to S phase transition after exposure to radiation, which allows cells additional time to

repair DNA (Brachman et al., 1991; Kastan et al., 1991; Querido et al., 1997; Ruaro et al., 1997; Shaulsky et al., 1991). In the case of mutant p53, cells are thought to accumulate genetic abnormalities because of the loss of G1 phase check point. p53 acts as dominant negative probably because mutant molecules interfere with formation of wild type tetramers and oligomers (Horikawa and Oshimura, 1991; May, 1993; Weinberg, 1991). The mutant p53 allele is transmitted through the germ line, leading to the familial Li-Fraumeni syndrome, that predisposes affected individuals at a young age to the variety of tumors. An animal model of p53 knockout supports the role of P53 in maintaining the intact genome, since homozygous mutants spontaneously develop cancers by the age of 6 months (Eng et al., 1997; Kleihues et al., 1997).

2B. Commonly found chromosomal translocations.

Nearly one third of ALLs and AMLs, two thirds of non-Hodgkin's lymphomas and nearly all CML cases are associated with the balanced chromosomal translocations without the loss of any genetic material (Yunis and Tanzer, 1993). Many of these translocations effect transcription factors of known and unknown properties. Identification of common chromosomal translocations in malignancies led to identification of many novel transcription factors. Chromosomal translocations are now used as markers for hematologic malignancies that help morphologic and histochemical characterization of diseases and allow a more precise diagnosis and treatment of patients. The more commonly affected

transcription factors in hematologic malignancies are HLH proteins (MYC, E2A) (Cameron et al., 1996; Jenkins et al., 1997; Lee et al., 1996; Perez-Stable et al., 1997), homeodomain proteins (PBX 1, HOX11) (Kamps et al., 1990; Kennedy et al., 1991) and Zinc-finger transcription factors (RARa, PLZF) (Chen et al., 1993; Dong et al., 1993).

2B-1. Burkitt lymphoma - a model for transcriptional deregulation of a bHLH/leucine zipper protein.

Burkitt lymphomas carry one of three chromosomal translocations, that move c-myc on chromosome 8q24.1 next to immunoglobulin loci at 2p11.1, 14q32.3 or 22q11.2. 2p11.1 involves Igl light chain and is found in 5% of all patients, 14q32.3 involves Ig heavy chain and comprises 80% of patients, while 22q11.2 effects Igκ light chain and is found in 15% of patients (Croce and Nowell, 1985; Yunis and Tanzer, 1993). Breakpoints in t(8;14) occurs 5' to the first exon or intron of the myc gene and results in a normal protein that is abnormally regulated. Transgenic mice with t(8;14) product expression develop B cell lymphomas (McDonnell and Korsmeyer, 1991).

2B-2.Philadelphia (Ph) chromosome in CML and ALL.

Ph chromosome was one of the first recurrent chromosomal abnormalities described in neoplasms by Dr. Janet Rowley (Rowley, 1973). Philadelphia

chromosome is formed as a result of the translocation of the abl gene on chromosome 9 to the bcr gene on chromosome 22 (t(9;22)). Translocation t(9;22) is found in 95% of CMLs, 20-30% of ALL in adults and up to 5% ALL in children (Berger et al., 1990; Bloomfield et al., 1977). Most of the breaks in the 230-kb long abl gene occur between exons 1a and 2. In the bcr (breakpoint cluster region) gene most of the breakpoints occur in the central part of the 160-kb gene between exons named b1 to b4 (corresponding to exons 12 to 15 of bcr gene). bcr always fuses to abl at intron 1, leaving abl under the control of the bcr promoter. The bcr-abl gene almost always contains 5' 13 or 14 exons of bcr fused to abl exons 2 to 11, resulting in the 8.5 kB mRNA that codes for a 210 kDa chimeric protein. In a small proportion of Ph chromosomes the first exon of bcr is fused to exon 2 of abl producing 7.0 kb mRNA that codes for 185 kDa fusion protein (Heisterkamp et al., 1985). All chimeric BCR-ABL proteins contain the DNA-binding domain of ABL along with three conserved SH1, SH2 and SH3 domains, with SH1 domain having properties of the tyrosine kinase (Pendergast et al., 1993; Pendergast et al., 1991). BCR contributes two SH2 domains and GTPase domain in p210BCR-ABL (Muller et al., 1993; Pendergast et al., 1991). BCR-ABL demonstrated transforming potential in cell culture system. When retrovirally transduced bone marrow cells expressing BCR-ABLp210 are transplanted into irradiated mice they develop CML and other hematologic tumors (Daley et al., 1991). Transgenic mice with p185 BCR-ABL develop ALL and die shortly after birth (Heisterkamp et al., 1990). These *in vivo* experiments provided a direct link between the product of chromosomal translocation and the etiology of the disease.

2C. Acute Promyelocytic Leukemia associated with disruption of Retinoic Acid Receptor (RAR) and Promyelocytic Leukemia (PML) genes.

Acute promyelocytic leukemia (APL) constitutes 10% of the cases of acute myeloid leukemia (AML) and is characterized by clonal expansion of myeloid cells blocked at the promyelocytic stage of development (Clarkson, 1991; Fenaux et al., 1993; Frankel, 1993; Grignani et al., 1994; Warrell et al., 1993). The promyelocytes contain bilobed nuclei, large azurophilic granules and Auer rods that characterize leukemia of the M3 subtype according to French American British classification. The primary azurophilic granules are full of procoagulants and when released activate coagulation cascade, deplete fibrinogen, clotting factors and platelets. As a consequence there is a high incidence of early mortality from intracranial bleeding (Grignani et al., 1994; Warrell, 1996; Warrell et al., 1993). Over 90% of APL cases are associated with reciprocal translocation t(15;17)(q22;q12-21), which fuses the Retinoic Acid Receptor α (RARα) on chromosome 15 to a promyelocytic leukemia (PML) gene on chromosome 17. Expression of t(15;17) is unique for APL and is usually not associated with any additional chromosomal abnormality. The breakpoint on the chromosome 17 always falls in the second intron, while the chromosome 15 breakpoint has three hot spots in the 3' portion of the gene that lead to variable C-termini in PML (Figure B). Translocation t(15;17) leads to formation of three aberrant proteins: PML-RARα, RARα-PML and a C-terminal truncated PML that arises from alternatively spliced PML-RARα with misaligned Open Reading Frames (ORFs) (Pandolfi et al., 1992;

Pandolfi et al., 1992). Identification of RARα's involvement in t(15;17) coincided with successful treatment of APL patients with All-trans Retinoic Acid (ATRA) in Shanghai, China (Huang et al., 1987). ATRA treatment leads to neutrophilic differentiation of APL cells in vitro and to remission in 84% of APL patients according to the recent clinical studies. Remissions achieved after treatment with just ATRA are short, lasting from 1 to 23 months. The best clinical predictor for the course of the disease is the presence or absence of PML-RARα RNA transcript in the bone marrow of patients. After remission the reappearance of the transcript, as demonstrated by RT-PCR, predicts a forthcoming relapse of the disease (Warrell, 1996; Warrell et al., 1993). Today a combination therapy of ATRA and chemotherapy leads to significant improvement in prognosis with 75-80% of patients undergoing complete remission.

2C-1. Retinoic Acid Receptor

Retinoic acid (RA) is known to play an important role in differentiation of many endodermally, ectodermally and mesodermally derived tissues. RA induces myeloid differentiation in vitro and may function through several classes of receptors. Retinoic Acid Receptors (RARs) are members of the steroid/thyroid nuclear receptor superfamily (Chambon, 1996; Napoli, 1996). There are three major subtypes of RARs, encoded by three different genes, RAR α, β and γ, which have different tissue distributions (Warrell et al., 1993). The primary sequence of the RAR protein is divided into six domains : A through F (Figure C). Domain C, consisting of 68 amino acids,

is the most conserved domain and is responsible for DNA binding. It contains two Cys2Cys2 Zinc-fingers (Zn-fingers), very similar to the Zn-fingers of estrogen (ER) and glucocorticoid receptors (GR). The DNA binding properties of RAR differ from ER and GR, which bind to palindromic sequences as homodimers. In contrast, RARs form heterodimers with Retinoid X Receptors (RXRs) and bind to Direct Repeat (DR) DNA sequences (Gaub et al., 1992; Leroy et al., 1991). The RXR family includes RXRα, β and γ and are distantly related to RARs in protein sequence. Heterodimers of RAR/RXR and RXR homodimers bind to retinoic acid response elements (RAREs) that are found in the promoters of rarβ2, crbp-1, laminin B1 and hox 1.6 genes among others. Domain E confers specificity of ligand binding to nuclear receptors. All-trans retinoic acid (ATRA) is a preferred ligand for RARs, while 9-cis RA is selectively bound by RXRs (Retinoid X Receptors), which are also members of the nuclear receptor superfamily (Kliewer et al., 1992; Mangelsdorf et al., 1992; Mangelsdorf et al., 1993). Domains A and B of RARs were demonstrated to have promoter-specific, ligand-independent transcription activating functions, while domain E has ligand-dependent transcriptional activation function (Nagpal et al., 1993; Nagpal et al., 1992). Each subclass of RARs and RXRs has several isoforms that are generated by alternative splicing of the 5' region of the genes and alternative promoter usage.

2C-2. Role of RARs in myeloid differentiation.

The pattern of RARα expression is ubiquitous and was detected in all hematopoietic cell lines examined. The steady-state level of RARα mRNA is not effected by differentiation of HL-60 cells, it also does not change in lymphocytes, whether they actively proliferate or rest (Kizaki et al., 1990). The role retinoic acid receptors play in myeloid differentiation was suggested by experiments of Breitman *et al.* in which they demonstrated that all-*trans*-retinoic acid and 13-*cis*-retinoic acid induce differentiation of promyelocytic HL-60 cell line (Breitman et al., 1980). The evidence for the crucial role of RARs was supported by existence of several differentiation resistant HL-60 cell clones, that contained C-terminally truncated RARα. Li *et al.* identified a single base change in codon 411, which leads to premature protein truncation and was the same mutation previously observed by Kizaki *et al* in the independently derived RA-resistant clone of HL-60 (Kizaki et al., 1990; Li et al., 1997). Interestingly, differentiation of the resistant clones of HL-60 cells could be restored by infecting cells with RARα, β or γ isoforms or with RXRα. These experiments suggest that RAR is indispensable for myeloid differentiation of HL 60 cells and indicate that retinoic receptors have functional redundancy. When a dominant negative RARα containing a mutation in codon 403 was introduced into normal bone marrow cells, these cells could not differentiate in response to GM-CSF, exhibited polyclonal expansion and developed into immortalized GM-CSF dependent cell line with morphologic appearance of promyelocytes (MPRO) (Collins et al., 1990; Robertson et al., 1992; Tsai et al., 1992; Tsai and Collins, 1993).

2C-3. Promyelocytic Leukemia (PML) protein.

The PML gene is approximately 35 kb long and contains nine exons, which are alternatively spliced to produce 13 isoforms. On the basis of its homology with other proteins PML is predicted to have a proline-rich N-terminal region, a cysteine-rich RING finger and B boxes, an α-helical region with a Leucine zipper-like segment and a serine and proline-rich carboxy-terminus (Fagioli et al., 1992) (Figure B).

PML contains three clusters rich in cysteines and histidines that are present in all PML and PML-RARα isoforms. The first cluster is a RING zinc finger motif hypothesized to function as a DNA-binding or protein -protein interaction motif. The RING finger motif is also found in RAG-1 (participates in DNA recombination), RAD 18 (DNA repair after UV-damage) and RPT-1 (effects IL-2 expression. Ring finger has been identified in breast cancer susceptibility gene with one of the cancer predisposing mutations affecting the RING finger (Futreal et al., 1994; Miki et al., 1994). PML also contains an additional two putative zinc finger motifs called B boxes, that are also found in T18 and rfp genes, known to be effected by chromosomal translocations associated with cancer. Biophysical studies including optical spectroscopy demonstrated that correct folding of the RING finger is necessary for proper nuclear localization of PML(Borden et al., 1995).

Some biological functions of PML have been elucidated recently. PML was shown to be a growth suppressor capable of inhibiting anchorage-independent growth of APL-derived NB4 cells in soft agar and suppressing transformation of NIH 3T3 cells by activated *neu* oncogene. It was also shown to suppress the transformation of REF cells by the combination of Ha-ras with c-myc or p53 (Mu et al., 1994; Mu et al., 1996). Le *et al.* analyzed the function of various domains of PML in growth and transformation suppression (Le et al., 1996). The results showed that RING and B box, NLS and dimerization (α-helical region) are required for growth suppression by PML and for localization to PML oncogenic domains (PODs) (Le et al., 1996). These results suggest the significance of correct subcellular localization for PML function. PML normally has speckled nuclear localization in PODs (even though some cell cycle variations has been documented (Koken et al., 1995)), while PML-RARα has a micropunctate nuclear pattern. In APL cells, which express wild type and mutant PML proteins, a micropunctate pattern of subnuclear localization is observed. This pattern reverses to PML-like speckled after treatment of cells with ATRA, again supporting the significance of correct PML localization for normal cell growth (Daniel et al., 1993; Dyck et al., 1994; Kastner et al., 1992; Weis et al., 1994).

2C-4. PML-RARα fusion protein acts differently from the wild type PML and RARα proteins.

PML-RARα protein invariably includes the putative DNA-binding region and dimerization region of PML and regions B though F of RARα, containing DNA binding and dimerization domains of RAR. Because of its structure PML-RARα is thought to inhibit function of the wild type PML and RARα in a dominant negative manner. PML-RARα displaces PML from its normal subnuclear localization in the PODs. Immunofluorescence demonstrated that PML is localized in the nucleus in a few large spherical structures. In contrast, PML-RARα fusion protein forms hundreds of small cytoplasmic and nuclear spherical structures in transiently and stably transfected cell lines. This pattern changes to resemble the wild type PML distribution of several nuclear domains after treatment of cells with RA (Daniel et al., 1993; Dyck et al., 1994; Kastner et al., 1992; Weis et al., 1994). The retargeting effect of RA on PML-RARα distribution correlates with the ability of RA to differentiate cells and suggests a dominant negative effect of PML-RAR on the wild type PML protein (Daniel et al., 1993; Kastner et al., 1992). This led to the hypothesis that PML within nuclear bodies has a growth suppressive effect.

PML-RARα can also interfere with the normal function of RARα, because in contrast to RARα it can form homodimers or heterodimers with RXR

(Pandolfi et al., 1991; Perez et al., 1993). PML-RARα binds to RAREs with different specificity and affinities from RAR/RXR heterodimers. PML-RARα also functions as a repressor in the absence of RA and as a better activator than RARα in the presence of RA (de The et al., 1991; Perez et al., 1993). In other systems, however, PML-RARα was shown to lack the transcription activation effect of RARα in the presence of ATRA (Licht et al., 1996). The ability to bind the same DNA binding sites with different affinity and to exert different transcriptional activities from the wild type protein may underlie the mechanism of PML-RARα dominant effect. At the same time, PML-RARα heterodimerizes with RXR and forms stable RARE-bound complexes. By sequestering RXR, PML-RARα interferes with the function of several members of nuclear receptor superfamily like RARs, Thyroid Receptor and Vitamin D3 receptor (Berrodin et al., 1992; Bugge et al., 1992; Hallenbeck et al., 1992; Kliewer et al., 1992; Kliewer et al., 1992; Marks et al., 1992). Aberrant DNA binding and sequestration of RXR are probably two main mechanisms by which PML-RARα interferes with normal cell growth.

2C-5. PML-RARα interferes with cell differentiation.

There is strong evidence suggesting that PML-RARα causes the differentiation block observed in APL cells. Expression of PML-RARα in K562 erythroleukemia cells leads to inhibition of erythroid differentiation, associated with reduced expression of differentiation markers and reduced responsiveness to differentiating agents (Grignani et al., 1995). When PML-RARα was expressed in U937 cells it also led to the block of

differentiation in response to Vit D3 and Vit D3 with TGF-β1 combination (Rousselot et al., 1994; Testa et al., 1994). Grignani *et al.* demonstrated that block to terminal differentiation in U937 cells fully depends on expression of the fusion between PML protein dimerization domain and RARα DNA binding domains (Grignani et al., 1996).

2C-6. Transgenic expression of PML/RARα in mice.

Several groups tried to recapitulate Acute Promyelocytic Leukemia in PML-RARα transgenic mice with variable effect on hematopoiesis. One of the first results came from Early *et al.*, who expressed PML-RARα under the control of CD11b+ promoter, which directs expression of a transgene in myelomonocytic cells (Early et al., 1996). The group did not observe any phenotype *in vivo*, except that the transgenic mice developed leukopenia after exposure to radiation. In vitro, peripheral blood from transgenic animals demonstrated a marked reduction in the number of myeloid progenitors in clonal growth assays (Early et al., 1996). Even though the expression of PML-RARα under CD11b+ promoter did not lead to leukemic transformation, there was an effect on myeloid development. A stronger effect was observed when PML-RARα transgene was expressed under control of human cathepsin G promoter (hCG) (Grisolano et al., 1997). hCG is active in early myeloid cells and at promyelocytic stage of differentiation. Transgenic mice had altered myeloid development with increased numbers of mature and immature myeloid cells in peripheral blood, bone marrow and spleen. Some of the cells had features of leukemic promyelocytes and responded to RA treatment with

differentiation. After a long latent period 30% of transgenic mice developed acute myeloid leukemia (Grisolano et al., 1997). Expression of PML-RARα transgene was probably not sufficient by itself to cause APL, but predisposed myeloid cells to leukemic transformation probably associated with additional genetic damage during the long latency period. Similar results were obtained by He *et al.* in transgenic mice, expressing PML-RARα under hCG promoter. They observed that all transgenic mice had myeloproliferative disorders and 10% of them developed APL responsive to ATRA treatment (He et al., 1997). Results from both groups directly implicate PML-RARα in pathogenesis of APL.

Brown *et al.*. expressed PML-RARα under the control of hMRP8 promoter and detected a phenotype consistent with the role of PML-RARα in oncogenic transformation in APL (Brown et al., 1997). Transgenic mice displayed a defect in neutrophil maturation early in life with development of APL within several months, consistent with a multistep progression of leukemia. Expression of PML-RARα transgene under the hMRP8 promoter that stays active during neutrophilic maturation, led to the block in myeloid maturation and to recapitulation of t(15;17) APL with differentiation in response to RA (Brown et al., 1997).

2D. APL associated with translocation t(11;17).

There are several varient chromosomal translocations that are associated with APL. Translocation t(11;17) was first identified in the study of 32 patients with APL by Chen *et al.* (Chen et al., 1993). The patient was found to harbor a rearranged RAR gene, translocated onto a novel gene, different from the PML gene. Chen *et al.*. identified 605 nucleotides of a novel Promyelocytic Leukemia Zinc Finger (PLZF) gene fused to RARα using anchored PCR with 'nested' primers for the RAR-B region (Figure C). Dr. Zelent subsequently screened a human ventricular muscle cDNA library with the novel sequence and obtained a 2.1 kb cDNA. The cDNA contains 2019 bp of open reading frame with an initiation codon at nt 1, surrounded by a Kozak consensus sequence for translational initiation (Chen et al., 1993). As of today there have been six patients identified with t(11;17). These patients have worse prognosis than patients with t(15;17), as they respond poorly to RA treatment and have high incidence of early death and dissiminated intravascular coagulation (Guidez et al., 1994; Licht et al., 1995).

2D-1. Structure of PLZF Protein.

There are two isoforms of PLZF: PLZF(A), that was originally cloned from the patient, and PLZF(B), that has an additional 123 amino acid fragment (nt759-1127), which is alternatively spliced (Figure C). The deduced

PLZF(B) amino acid (aa) sequence contains clusters of negatively charged residues and prolines in the amino-terminus, that are found in many transcriptional activation domains. The carboxyl-terminus, which is similar for both isoforms, contains nine *Kruppel*-like Zinc-fingers. The Zn-finger DNA binding motif is present in many factors that regulate eukaryotic gene expression. Such domains are found in transcription factors important for differentiation (EGR-1, MZF-1), in tumor suppressor genes (Wilms' tumor gene) and oncogenes (Gli) (Bavisotto et al., 1991; Nguyen et al., 1993; Sukhatme, 1992). The three Zn-fingers of EGR-1 were crystallized together with its consensus DNA binding site and it was shown that Zn-fingers bind in the major groove of B-DNA, bringing alpha helices in contact with a three base pair subsite (Pavletich and Pabo, 1991). The DNA sequence that is recognized by Zn-fingers of PLZF has been identified by Dr. Jia Li using a PCR-based binding site selection strategy. After two rounds of selection the optimal PLZF binding site was identified as TAAAGTTTGATCTGTTC. TAAA and GTTC are key DNA contact positions, since their mutation leads to severe decrease in affinity of PLZF to DNA (Li et al., 1997). The RARα-PLZF, which contains seven Zinc fingers of PLZF, can bind to the selected site with the same affinity as nine zinc fingers of PLZF. At the same time first two Zn-fingers, contained in PLZF-RARα reciprocal fusion protein, does not bind to the selected DNA site (Li et al., 1997). This data suggest that PLZF-RARα can interfere with the function of the wild type PLZF protein by binding to the same DNA sequences. Data presented by Li *et al.* demonstrates that PLZF represses transcription through its DNA binding site and that RARα-PLZF lacks this ability to repress transcription. Furthermore, it is very intriguing that the binding site for PLZF identified by Li *et al.* is present in Cyclin A2 promoter and that preliminary results

indicate that PLZF can inhibit cyclin A expression, while RARα-PLZF may upregulate cyclin A2 expression in 32DG/GM cells (Yeyati et al., 1997). These results suggest that Cyclin A2 is a biological target of PLZF and implicate PLZF in the control of the cell cycle. Another group discovered that an acidic transactivation domain fused to the last five Zn-fingers of PLZF bound to lex-A operator in two hybrid screening (Sitterlin et al., 1997). The core binding sequence in lexA operator is TATGTACAGTAC. The possible consensus binding site for PLZF after the alignment of independently selected sequences is **A**(T/G)(G/C)**T**(A/C)(A/C) **AGT**.

The amino terminus of PLZF contains a motif that is evolutionary conserved from pox virus to humans and is termed POZ (**POX**virus and **Z**inc finger) (Bardwell and Treisman, 1994). This domain is found in Bcl-6, the zinc finger protein KUP , Zinc Finger 5 (ZF5) mammalian proteins, Tramtrack (TTK) and Kelch of Drosophila and several viral proteins (Bardwell and Treisman, 1994; Numoto et al., 1993; Zollman et al., 1994). The POZ domain is approximately 120 amino acids long and is located in the N-terminus of the PLZF protein. The POZ domain has been shown to mediate self-association of the ZF5 and ZID proteins, and allow heteromeric interactions of TTK and GAGA proteins in *in vitro* assays. Careful studies of the ZID protein POZ domain showed that it can interfere with the DNA binding of several heterologous DNA binding domains (Bardwell and Treisman, 1994; Numoto et al., 1993).

Li *et al.* demonstrated that PLZF contains two repression and one activation domain (Li et al., 1997). PLZF(1-100aa) and PLZF (200-300aa) fused to a GAL4(1-147aa) DNA binding domain was able to repress transcription from a plasmid containing GAL4 binding sites in *in vivo* transfection experiments. PLZF (1-100aa) contains the POZ domain, which, besides attenuation of DNA binding, is probably involved in protein-protein interactions that lead to repression of transcription. It is significant that POZ is present in several Zn-finger proteins like ZF5, ZID, Bcl6 and PLZF that can repress transcription. A weak activation domain was mapped to amino acids 100-200 and was rich in acidic residues (Li et al., 1997). Thus, PLZF has all the necessary attributes of a transcription factor: DNA binding Zn-fingers; a POZ domain, that can repress transcription; an activation domain and nuclear localization of the protein (see Chapter IV).

2D-2. Tissue distribution of PLZF expression.

Northern blot analysis showed that PLZF is expressed in bone marrow, peripheral blood mononuclear cells, ovaries, lungs and kidneys (Reid et al., 1995). Expression of PLZF in HL-60 and NB4 cells is downregulated upon RA induced differentiation (Chen et al., 1993). PLZF is expressed in CD34+ bone marrow cells and in the IL-3 dependent myeloid progenitor cell line 32D, but not in more differentiated myeloid or erythroid cell lines. In CD34+ cells PLZF is localized to large nuclear speckles, that are reminiscent of PODs , that contain PML. It is possible that subnuclear localization is important for the function of PLZF and that t(11;17) may disrupt normal localization (Reid et al., 1995). Preferential expression of

PLZF in the immature hematopoietic cells suggests that it may play a role in the maintenance of hematoipoietic progenitors.

PLZF is also expressed in a specific spatio-temporal pattern in the CNS of the developing mouse. At 7.5 days post conception (dpc) mRNA can be detected in the anterior edges of the head folds, the rostral extremity of the neural fold formation and in the region of the caudal neuropore. At 10.5 dpc it could be detected in the brachial arches, limb buds and frontonasal mesenchyme. When rhombomeres become distinguishable at 9.0 dpc the mPLZF mRNA levels are downregulated in the center of r3 and r5, while maintaining high expression at the rhombomeric boundaries, and are high in r2,4,6 (Avantaggiato et al., 1995; Cook et al., 1995). The biological function of PLZF may well go beyond its role in hematopoiesis. The importance of PLZF in central nerveous system is suggested by the high degree of conservation between the pattern of PLZF expression in the developing mammalian and avian central nerveous systems and by its complex spatial and temporal pattern of expression (Cook et al., 1995).

2D-3. Transcriptional properties of PLZF-RARα and RARα-PLZF fusion proteins.

In all patients with t(11;17) the RARα gene is broken between exons 3 and 4, which code for the A and B domains respectively (Figures B and C). The breakpoint in the PLZF gene is within an intron between Zn-fingers two and

three in five patients, and in one patient between Zn-fingers three and four. The translocation effects only one allele of PLZF, while the second allele remains intact. As a result patients with t(11;17) express RARα-PLZF and PLZF-RARα proteins in addition to the wild type proteins. PLZF-RARα can form homodimers and heterodimers with RXR. PLZF-RARα homodimers bind DNA very weakly and probably do not play an important role in competition with RARα. On the other hand PLZF-RARα/RXR heterodimers bound to the RAREs DR2 and 5 with the same specificity and stability as wild type RARα (Chen et al., 1994; Licht et al., 1995). PLZF-RARα/RXR heterodimers exert a dominant negative effect over RARα/RXR, inhibiting the ability of RAR/RXR to activate transactivation from RAREs. The dominant negative effect depended on the presence of the POZ domain and did not require acidic region and the first two Zn-fingers of PLZF (Dong et al., 1996). This data suggest that PLZF-RARα may contribute to leukemogenesis by sequestering RXR and thus interfering with the function of nuclear receptors, and/or by binding to RAREs and altering transcription from RARa target genes. PLZF-RARα may also interfere with PLZF function by forming heterodimers with PLZF and diminishing the effective PLZF concentration in the cell (Licht et al., 1996). The experimental evidence suggest that PML-RARα and PLZF-RARα effect transcription from RA-responsive genes in a similar manner: binding to RAREs with different affinity, sequestering RXR and exerting dominant negative effect over wild type RARs. Nevertheless patients carrying t(11;17) have much worse prognosis and a disease that is not responsive to the differentiation therapy with RA. This suggests that the difference arises from disruption of PLZF and from the effect produced by RARα-PLZF on PLZF target genes.

RARα-PLZF contains transactivation domain A1 or A2 of RAR and seven Zn-fingers of PLZF, that were shown to bind to PLZF DNA binding site (Li et al., 1997). The transactivation domains would confer novel transcriptional properties to PLZF and may disregulate the PLZF target genes. Transient transfection assays performed by Dr. Jia Li and Dr. Patricia Yeyati have shown that RARα-PLZF lacks the transcription repression properties of wildtype PLZF and in some cases, RARα-PLZF can even activate target genes, one of which is currently hypothesized to be the cyclin A2 gene. RARα-PLZF may directly contribute to leukemogenesis by binding to the cyclin A2 promoter and activating cyclin A2 expression. This by itself should be sufficient to promote G1 to S transition and to transform cells (Barlat et al., 1993; Barlat et al., 1995; Yeyati et al., 1997).

Figure A. Schematic presentation of the stem-cell compartment. (THSC=totipotential hematopoietic stem cell; PMSC=pluripotential myeloid stem cell; CFUs=colony-forming unit-spleen; HIM=hematopoietic inductive microenvironment; CFU-TL= colony-forming unit-T lymphocyte; CFU-BL= colony-forming unit-B lymphocyte; CFUd= colony-forming unit-diffusion {chamber}; CFUnm= colony-forming unit-neutrophil-monocyte; CFUeos= colony-forming unit-eosinophil; BFUe=burst-forming unit-erythroid; CFUe= colony-forming unit-erythroid; CFUmeg= colony-forming unit-megakaryocyte; CFUbas= colony-forming unit-basophil.)

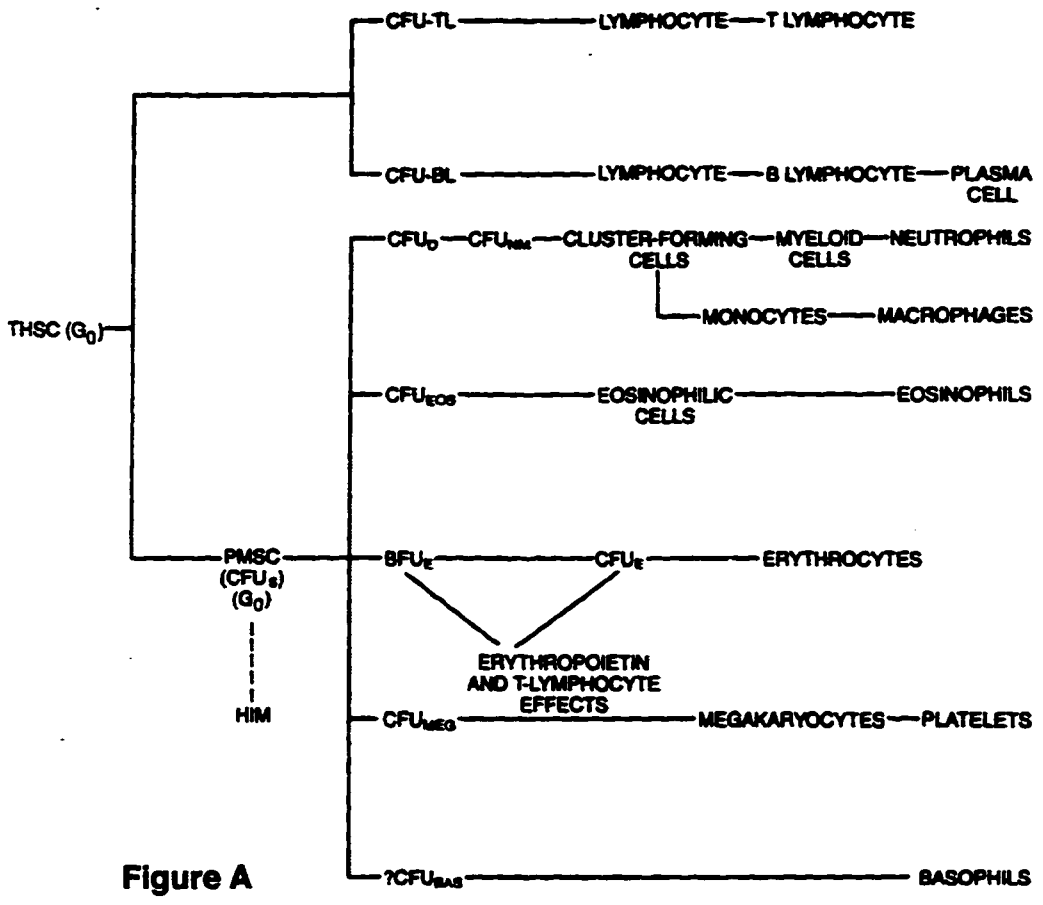


Figure A

Figure B. Schematic presentation of the wild type PML protein and its fusions with RARα. Wild type PML protein contains the following regions: N-terminal proline-rich region (P), the putative DNA-binding region (the ring domain {R} and two B boxes {B1 and B2}), dimerization domain with coiled-coil region (cross-hatched) and non-coiled-coil portion of the α-helix (blank) and the most C-terminal Ser/Pro-rich region. The non-coiled-coil portion of the α-helix is the site of translocation breakpoints that lead to the variable length of the fusion proteins PML-RARα(L) and PML-RARα(S). PML-RARα contains the portion of PML of variable length, which always includes proline rich region, DNA-binding region and a dimerization domain, and B through F regions of RARα, that include DNA-binding and dimerization domains. RARα-PMLs are reciprocal fusions of PML-RARα and consist of transactivating domain A of RARα and Ser/Pro rich region of PML, in addition to the variable segment of non-coiled-coil portion of the α-helix.

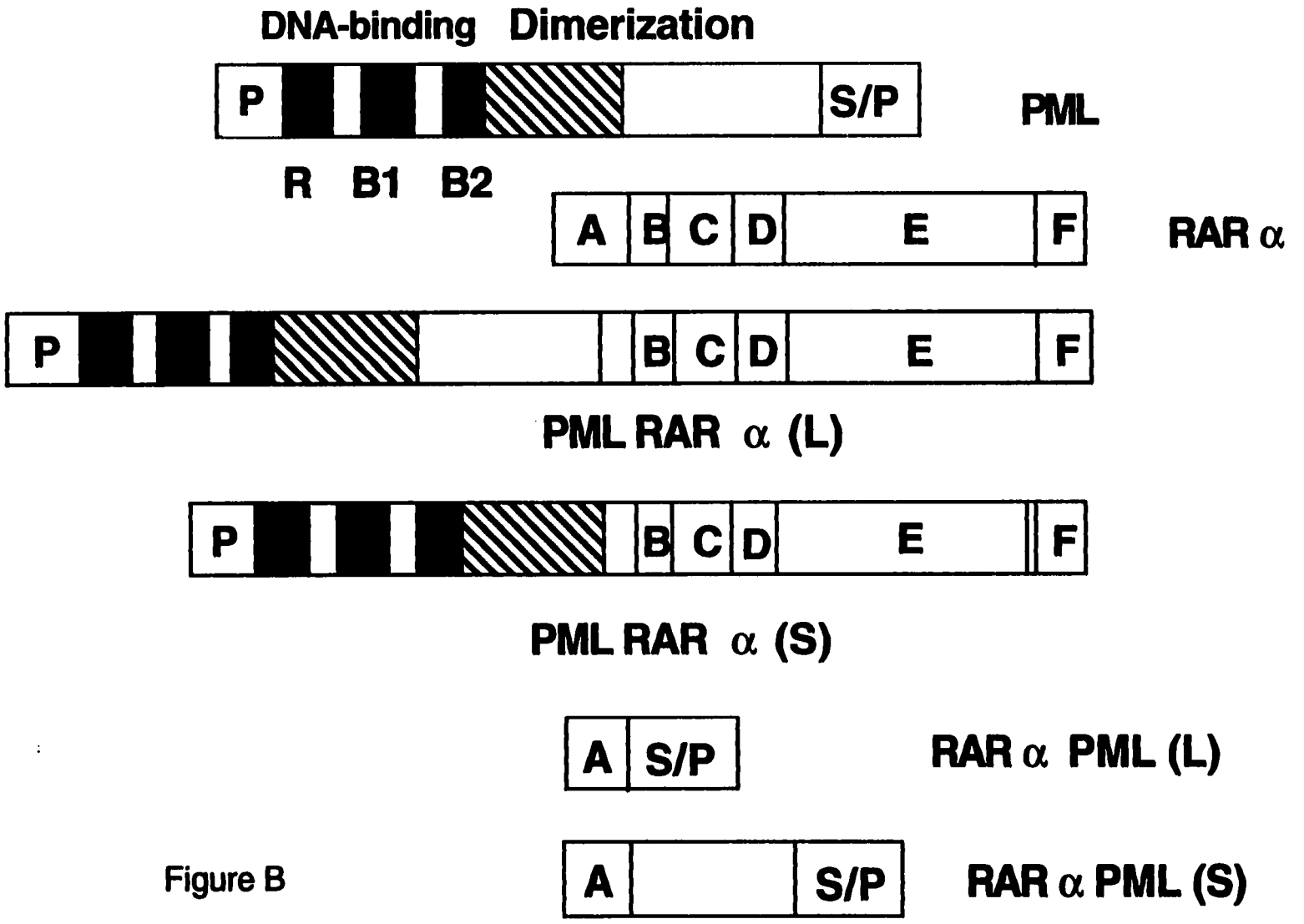


Figure B

Figure C. Schematic presentation of PLZF, RARα and their fusion proteins in t(11;17). The arrows indicate the sites where the fusion occurs. PLZF-RARα contains N-terminus of PLZF, including the POZ domain, acidic domain and its two N-terminal Zc-fingers and B through F domains of RARα, that include DNA-binding and dimerization domains. The reciprocal fusion protein RARα-PLZF contains A1 or A2 activating domain of RARα and seven C-terminal Zn-fingers of PLZF.

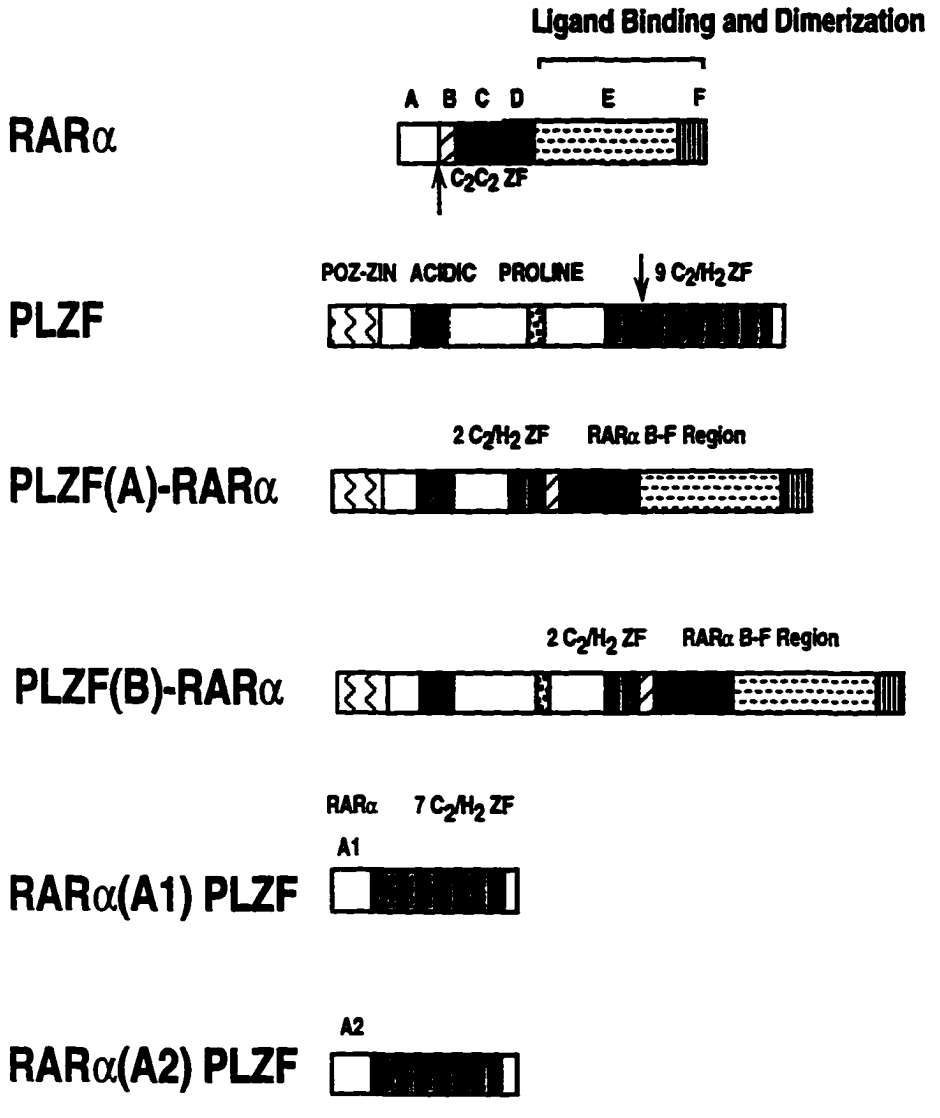


Figure C

Chapter II

Materials and methods

Plasmid Construction PLZF(A)-RARα, PLZF(B)-RARα and RARα1-PLZF fusion genes were expressed under the control of the SV40 promoter/enhancer included in the SG5 plasmid (Green et al., 1988) (Stratagene) (Chen et al., 1994) (Gifts of Z. Chen). The murine retinoic acid receptor α1 (RARα1) expression vectors were described previously (Leid et al., 1992; Zelent et al., 1989) (Gift of P. Chambon). A PLZF expression vector was constructed by Dr. J.Licht by insertion of the 2.1 kb *EcoR*1 fragment containing the PLZF cDNA (Chen et al., 1993) into pSG5. An epitope tagged version of PLZF, PLZF-Flag was constructed by insertion of a duplex oligonucleotide of the sequence:

5' GATCCGCCACCATGGACTACAAGGACGACGATGACAAA 3'

3' GCGGTGGTACCTGATGTTCCTGCTGCTACTGTTTCTAG 5'

into the *Bam*H1 site of pBluescript. The 2.1 kb PLZF cDNA (Chen et al., 1993) was inserted downstream of the oligonucleotide in the *Eco*R1 site of pBluescript. The resulting fusion protein contains the additional amino acids MDYKDDDKDPPGCRNS including the Flag epitope (IBI, New

Haven, CT), N-terminal to the first methionine of the PLZF protein. A PLZF-Flag expression vector was constructed by excising the PLZF-Flag cDNA from pBluescript by digestion with *Bam*H1 and *Sal*I. This fragment was inserted downstream of the rous sarcoma virus promoter (RSV) (made by Dr. J.Licht) (Haber et al., 1992). A GAL4-PLZF fusion protein was constructed by M.A.English by digesting a plasmid encoding the DNA-binding and dimerization domains of the yeast GAL4 protein (amino acids 1-147) (Gift of M. Ptashne) with BamH1 and Xba1 (Li et al., 1997). A fragment of the PLZF cDNA encoding amino acids 1-400 of PLZF was amplified by PCR using the N-terminal primer:

5' CGCGGATCCGTATGGATCTGACAAAAATG 3'and the C-terminal primer:

5' GCTCTAGAGCCGGCTCTCTGACTT 3'. The N-terminal primer was designed to allow in frame fusion to the GAL4 coding sequence and the C-terminal primer added one addition amino acid (glutamic acid) to the PLZF fragment before encountering an in frame stop codon. The resulting PCR fragment was disgested with BamH1 and Xba1 and ligated to the GAL4(1-147) plasmid which includes the SV40 promoter/enhancer. A similar PCR based strategy was used to make GAL4-PLZF(1-100), GAL4-PLZF(100-200) and GAL4-PLZF(100-400) by J.Y. Li. (Li et al., 1997).The sequence at the junction point between GAL4 and PLZF and of the entire PLZF segment was confirmed by DNA sequencing and no PCR generated errors were encountered. A plasmid to express the PLZF protein in bacteria as a fusion

protein with glutathione *S*-transferase, pGST-PLZF was constructed by insertion of a 2.1 kB EcoR1 fragment encoding the entire open reading frame of the PLZF into pGEX3X (Pharmacia) (Chen et al., 1993). pBabePLZFpuro vector was constructed by insertion of the 2.1 kb EcoR1 fragment containing PLZF cDNA (Chen et al., 1993) into pBabepuro (Morgenstern and Land, 1990). pGST-WT has WT1 cDNA in pGEX-2TK and was a present of Dr. Yang Shi. pSVneoHMTIIA contains human metallothioneine promoter and a neomycin resistence gene and was a present of Dr. Jim Bieker. Inducible PLZF-flag construct was cloned by ligating EcoR1 fragmant of PLZF-flag into the polylinker of pSVneoHMTIIA. PLZF-RARα and RARα-PLZF EcoR1 fragments were cloned into pSVneoHMTIIA in a similar manner. Another inducible vector EBOplpp was a present of Dr. Ethan Dmitrovsky and is an episomal vector induced to amplify by increasing concentrations of hygromycin. (ref) EBOplppantisensePLZF was constructed by digesting the vector with NotI and SalI and ligating NotI-SalI fragment of PLZF cDNA. The sense construct was made by cutting the vector with NotI and SalI. PLZF fragment was obtained by first cutting the pBSPLZF with NotI and then doing a partial digestion with KpnI. 2.213 kB PLZF containing fragment was ligated into EBOplpp vector.

Cell culture and morphological assessment. CV-1 African green monkey kidney cells were grown in DMEM containing 10% calf serum, 50 units/ml of pennicilin, 50 mg/ml of streptomycin, 2 mM glutamine (GibcoBRL) (pen/strep, glu). Myelodysplastic (MDS) cells (Banerjee et al., 1992) were grown in MEM/10% heat inactivated Fetal Bovine Serum (FBS; Hyclone)/ pen, strep/ glu.

The murine IL-3 dependent 32DCL3(G/GM) cell line was previously described (Kreider et al., 1990) The cells were grown in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL) supplemented with 10 % heat inactivated FBS/ pen,strep/ glu and 10 ng/ml of recombinant murine IL-3 (Genzyme,Cambridge, MA). Differentiation studies were done with 30 ng/ml of recombinant human G-CSF (R&D Systems, Minneapolis, MN) and 6ng/ml of recombinant mouse GM-CSF (Genzyme, Cam,bridge, MA). HL 60, U 937, NB4D5 and Hybridoma 2A9 were grown in RPMI/ 10% heat inactivated FBS/ pen, strep/ glu. The Ψ2 retroviral packaging cell line was maintained in DMEM supplemented with 10% FBS/ pen, strep/ glu. The serum was heat inactivated by heating at 56^0C for 30 minutes. To study the effect of conditioned medium on the growth of 32DG/GMVector cells, cells were plated at a density of $1x10^5$ cells/ml with or without 50% conditioned medium. The medium was conditioned by plating control or PLZF expressing cells at a density of $1x10^4$ cells/ml and removing the medium after 3 days or by removing conditioned medium from exponentially growing cells where indicated. The effect of IL -3 withdrawal on growth and

survival was studied by washing cells twice with 5 mls of PBS followed by incubation in complete medium without IL-3 supplementation. For morphological charactrerization cells were collected, washed and spun onto polyLysine slides in 1%BSA/PBS (Shandon, Sewickly, Pa.), after which they were stained with modified Giemsa stain (Sigma Diagnostics, St.Louis, Mo.) All slides were coded to eliminate bias and counted at least twice .

Generation of the stable cell lines. Stable lines were generated using retroviral infection. To create the packaging lines ψ2 cells were electroporated with 10μg of NotI linearized pBabe or pBabePLZF at 300V and 250mF. The transduced cells were selected with 1.5 mg/ml of puromycin for two weeks and individual clones were isolated. The supernatant from packaging clones was screened by its ability to confer puromycin resistance to 3T3 cells. The positive clones were expanded and screened by immunoblotting with anti-PLZF antibody for expression of PLZF. $5x10^6$ 32D CL 3(G/GM) cells were co-cultivated with the supernatant overnight, removed and allowed to grow. After 48 hs they were selected in 1.5mg/ml of puromycin for 2-3 weeks.

Transfections. CV1 or COS-7 cells were grown in a 5% CO_2 environment in DMEM supplemented with penicillin/streptomycin, 10% calf serum or 10% delipified, charcoal-stripped calf serum. One day before

transfection the cells were plated at a density of 10^6 cells per 100 mm tissue culture plate. The plasmids were transfected in amounts indicated in the figure legends along with 0.5 μg of pMTGH (Selden et al., 1986), a human growth hormone (hGH) expression plasmid, as an internal control of transfection efficiency. Transfections were performed by the calcium-phosphate precipitation method as described previously (Licht et al., 1994). Chloroquine (50 μM) was added to cell media along with the calcium phosphate precipitate and incubated with the cell monolayers overnight. At 48 hours post-transfection, cell culture media were harvested for hGH radioimmunoassay (Allegro, San Capistrano, CA) according to the manufacturer's instructions. Cells were harvested and whole cell extracts were prepared as described (Ausubel et al., 1989).

Polyclonal Rabbit and Monoclonal Mouse Antibodies production. To generate antibodies to the PLZF protein, HB101 bacteria were transformed with the GST-PLZF plasmid and protein was induced as described, (Ausubel et al., 1989), (Smith and Johnson, 1988). GST-PLZF fusion protein was collected from bacterial extracts on glutathione agarose beads (Sigma) and eluted with 20mM gluathione, 100mM Tris, pH 7.5. The GST-PLZF fusion protein (200 μg) was injected subcutaneously into a rabbit after emulsification with Freund's adjuvant (Harlow and Lane, 1988) by Dr. T.Moran from the Hybridoma and Cell Center Core Facility at Mount

Sinai. Subsequent booster injections of 200 μg of fusion protein were performed 2 and 4 weeks after the initial immunization. Serum was isolated from whole blood and antibodies against PLZF were purified through a column of GST-PLZF covalently linked to sepharose as described (Harlow and Lane, 1988).

Hybridoma cell lines that secreted monoclonal mouse antibodies against PLZF were made by Dr. T. Moran. We recieved 6 hybridomas: five of them were of IgG1 isotype (3G1, 37A8, 47C6, 17B10, 31D10) and one was of IgG2A isotype (2A9). After screening hybridoma supernatants hybridoma 2A9 prooved to have higher specificity and sensitivity for PLZF. Antibodies were purified from 500 ml of hybridoma 2A9 supernatant by modified low salt perfusion chromatography using Protein A column (Harlow and Lane, 1988). All purification steps were done in the cold room (4°C). After 500 mls of hybridoma supernatant were run through the column it was washed with 300 mls of Phosphate Buffer Saline (PBS), pH 7.0 until the protein level in the flow through was 0, based on the O.D. at 280 nm. After that the antibodies were eluted with 0.1M Sodium Citrate, pH3.5 and collected into 1.5-ml conical tubes containing 50μl of 2M Tris, pH 10.45 as a buffer. Protein concentration of all fractions was measured using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Fractions with highest protein concentrations were pooled together and dialyzed in four liters of PBS overnight at 4°C. After that the protein concentration of antibodies was

measured again, antibodies were aliqouted into 100µl fractions, frozen in liquid nitrogen and stored at -20°C.

Immunostaining and flow cytometry. Immunostaining was performed on 10^3-10^6 cells per sample, depending on the cell line and cell availability. Cell surface staining was performed first, followed by the intranuclear antigen staining and lastly by DNA staining when necessary. 32D CL3G/GM cells were first blocked in 1xPBS/2%FBS/2%mouse serum for 30 min at room temperature, followed by addition of the primary antibody against the cell surface antigen and incubation for 30 min. The cells were then washed three times with 1XPBS/2%FBS. Secondary antibody was added only if cell surface staining was performed. When intranuclear staining was performed cells were permeabilized by incubation in 0.2%Tween/1xPBS for 15 min at 37°C, followed by incubation with the antibody against the intranuclear antigen for 30 min. Next cells were washed three times in 1xPBS and incubated with the appropriate secondary antibody conjugated to the fluorochrome for 30 min and washed three more times with 1xPBS. All antibodies were diluted in the blocking solution and all incubations were carried out in 100 µl volumes at room temperature. For DNA staining, cells were incubated with 10mg/ml of propidium iodide in 1xPBS and DNAse free RNAse

(Boehranger Manheim, Indianapolis, In) for at least 30 min. Samples were kept at 4°C for up to 1 day and then analyzed.

Antibodies. Polyclonal rabbit (PR) anti-mouse IL-3 receptor antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was used at 300 ng/ml; PR IgGs were purchased from R&D System; rat anti-GR-1 Phycoerythrin (PE) , rat anti-Sca-1 PE and rat IGg2b, kappa PE conjugated were purchased from PharMingen (San Diego, CA) and were used at 0.2 mg/sample. Monoclonal mouse anti-PLZF antibody was raised inMount Sinai core facility and was used at 1mg/ml. Anti-RARα1 (A1 region) antibody was used at 1:500 dilution for the Western Blot and was a gift of Dr. Chambon (Rochette-Egly et al., 1991).

Flow cytometric analysis of apoptosis. Apoptosis was assessed by a modified TUNEL Assay (Apotag, Oncor, Gaithersburg, MD). In short, cells were fixed in 1% paraformaldehyde, followed by 70% ethanol. After this the free 3OH' ends of DNA were extended with digoxigenin-labeled nucleotides using terminal transferase and stained with fluorescein-conjugated anti-digoxigenin antibody. Fluorescent emission was recorded using FACscan flow cytometer and analysed using LYSIS software (Nippon Beckton Dickinson). Another approach for the assessment of

apoptosis was based on the ability of Annexin V to recognize phosphoserines on the surface of the dying cells (ApoAlert Annexin V Kit, Clontech Laboratories, Palo Alto, CA). Live cells were stained with PI and Annexin V conjugated FITC, washed and analysed. PI positive cells representing necrotic cells were gated out and PI negative cells were analysed for the FITC staining on FL1 channel using LYSIS software.

Immunofluoresence. For immunofluoresence studies, CV1 cells grown in DMEM containing 10% charcoal-stripped calf serum were transfected with RSV-PLZF-Flag, SV40-PLZF(A)RARa or SV40-PLZF(B)RARa or SV40-RARα-PLZF. Cells were treated with 10^{-6} M ATRA as indicated. The transfected cell monolayers were washed twice with ice-cold PBS, fixed for 5 minutes in 100% methanol at -20°C and blocked with 3% bovine serum albumin in PBS for 20 min. The cells were then washed three times for 5 minutes with PBS and cells within a circumscribed area of the tissue culture dish were incubated with rabbit polyclonal anti-PLZF antiserum at 1:1000 dilution for 45 min. The cells were then washed three times with PBS and incubated in the dark with a 1:2000 dilution of fluorescein-conjugated goat-anti rabbit IgG (Boehringer-Mannheim) in 3% BSA/PBS. The cells were washed five times with PBS and covered with several drops of 2.5% 4, diazabicyclo{2.2.2}-octane in glycerol and 10mM

Tris, pH 7.6 as an anti-fading agent. Cover slips were applied and sealed in place with clear nail polish. Cells were visualized under phase contrast and fluorescent microscopy using a Zeiss Axiophot microscope. HL-60 cells were grown in RPMI 1640 containing 10% fetal calf serum. These cells were immobilized on poly-lysine coated glass slides using a Cytopsin 2 (Shandon, Chesire, United Kingdom) fixed as above and stained with anti-PLZF antisera as above. Myelosdysplastic (MDS) cells were grown in DMEM containing 10% calf serum. PLZF and PML protein expression in MDS cells was examined before and after treatment of the cells with 400 nM calcium ionophore A23187 (Sigma, St. Louis, MO) for 48 hours. The cells were spun onto microscope slides, fixed as above and stained with anti-PLZF antisera (1;1000), anti-PML polyclonal antisera (1:500) (Gift of P. Chambon) or pre-immune rabbit sera. Laser scanning confocal microscopy was performed using a Leica system. (Leica, Germany

Immunoblotting. Whole cell extracts from CV1 cells tranfected with PLZF-RARα, PLZF or RARa-PLZF expression vectors were prepared by boiling cells in 1X sample buffer (6.25 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) followed by centrifugation for 5 min at 13,000 x g at room temperature. Fractionation of cells into nuclear and cytoplasmic extracts was performed as described (Andrews and Faller,

1991), lysing cells in hypotonic saline, separating the nuclear pellet and mixing each fraction with an equal volume of 2x SDS loading buffer. After boiling in sample buffer, cellular proteins were separated on 12% or 15% SDS polyacrylamide gels and transfered to Immobilon PVDF membrane (Millipore, Bedford, MA) in 192 mM glycine, 25 mM Tris base buffer overnight at 25 V. The filters were blocked in 5% nonfat milk dissolved in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) for one hour and washed 3 times for 5 minutes with 0.5% nonfat dry milk in TBST. The filters were then incubated with a 1:1000 dilution of anti-PLZF polyclonal rabbit antisera or affinity purified anti-PLZF antibodies at a concentration of 0.5 μg/ml for 1 hour. To detect the RARα-PLZF fusion protein a 1:500 dilution of a monoclonal antibody directed against the A1 domain of RARα (Gaub et al., 1992) was used in a similar manner. The filters were washed 3 times for 5 minutes with TBST and incubated with a 1:7500 dilution of goat anti-rabbit IgG or goat anti-mouse IgG coupled to horseradish peroxidase (Boehringer-Mannheim, Indianapolis, IN) for one hour, followed by 5 washes with TBST. Immunoreactive proteins were visualized by chemiluminescence and autoradiography (ECL Kit, Amersham, Buckingshamshire, United Kingdom).

Cell labelling and immunoprecipitation. CV-1 cells were transfected with the 10 μg of the PLZF-Flag expression vector or as a

control a SV40-fos expression vector (Lloyd et al., 1991) (Gift of A. Lloyd) as above. At 40 hours post transfection the cell monolayers were washed in either phosphate free media or methione-free media three times and then grown in phosphate or methionine-free media containing dialysed calf serum for three hours. The cells were then metabolically labeled with 10 μCi of [^{35}S] methionine or 2 mCi of [^{32}P] orthophosphate for three hours. The excess media was removed, the cells were washed three times in PBS and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 1 μM sodium orthovanadate, 1 mM NaF, 2 μg/ml leupeptin , 1 μg/ml antipain, 5 μg/ml aprotinin, 1 μg/ml bacitracin, 50 μg/ml soybean trypsin inhibitor, 1 mM benzamidine) for 30 minutes on ice. The extracts were homogenized by passage through a #22 gauge needle and centrifuged for 10 minutes at 20,000 x g at 4°C to remove cellular debris. Immediately thereafter, 250-400 μl of each extract was incubated wth 3 μg of M2 monoclonal anti-flag antibody (IBI, New Haven, CT) or 1 μg monoclonal anti-fos antibody (Santa Cruz Immunologics, Santa Cruz, CA) overnight at 4°C. The immunoprecipitates were collected by addition of 100 μl of a 10% slurry of staph A beads in lysis NP-40 lysis for one hour at 4°C. The beads were collected by brief centrifugation and washed washed 5 times in NP-40 lysis buffer. Immunoprecipitated proteins were released by heating at 95°C for 3 minutes in 2x SDS loading buffer, and were electrophoretically separated through a 10% SDS polyacrylamide gel. To detect [^{32}P] leabeled proteins, the gels were fixed in 30% methanol, 10% acetic acid,

dried and autoradiographed at -80°C with an intensifying screen. To detect [^{35}S] labeled proteins, the gels were fixed, then soaked for 1 hour in distilled water, 1 hour in 1M sodium salicylate dried and fluorographed at -80°C.

Graphics Autoradiographs were imaged using a Silverscan flatbed digital scanner (La Cie, Ltd., Beaverton, OR). Ektachrome slides of immunofluorescence experiments were imaged using a 35mm Rapid Film Scanner (Kodak, Rochester, NY). Image processing was performed on a Macintosh 7100 or 7300 (Apple, Cupertino, CA) using Adobe Photoshop (Adobe, Mountain, CA) and Aldus Persuasion (Aldus, Seattle, WA). Figures were printed using a XL7700 Digital Continous Tone Printer (Kodak, Rochester, NY) or Tektronix printer.

Chapter III

Production and characterization of the antibodies against human PLZF.

INTRODUCTION

After the*PLZF* gene was identified as a result of the t(11;17) associated with APL, the interest in the biological role of the new gene became very high. In order to answer questions about the biology of PLZF protein, about its pattern of expression and its biological functions we needed to raise antibodies against PLZF. Polyclonal rabbit and monoclonal mouse antibodies were raised in the Hybridoma Core facility of the Mount Sinai Medical Center using protein preparations that Dr. Jia Y.Li and I provided. Antibodies are essential tools for the study of the proteins, but for optimal use their properties must be characterized. In my characterization of the anti-PLZF antibodies I addressed such parameters as specificity and sensitivity of antigen recognition and determined conditions for such techniques as the Western Blot and Immunoprecipitation.

RESULTS

Analysis of the Polyclonal Rabbit Antisera raised against PLZF. We received the antiserum from three different rabbits immunized with GST-PLZF. Dot-blot analysis demonstrated that out of three tested antisera, #3 had the highest specificity for PLZF (reacted more avidly with GST-PLZF, than with GST and GST-WT) and sensitivity (was able to detect 0.5ng of GST-PLZF with 1:100 dilution and reacted more strongly than antisera #1 and #2 with 5ng of GST-PLZF) (Figure 1). All three antisera recognize GST and GST-WT proteins, since they contain a fraction of antibodies specifically recognizing a GST epitope. Based on the results of the dot-blot analysis we decided to continue our work with antiserum #3. In order to obtain a reagent of higher purity and concentration I also tested the IgG fraction from antiserum #3 (purified by the Core Facility) and the affinity purified fraction against GST-PLZF. The IgG fraction probably had improved purity and defined concentration, but as expected still reacted with GST and GST-WT (Figure 2A). This reagent contains only class G antibodies and has superior qualities than polyclonal serum when used for immunostaining of cells. On the other hand, affinity purification of antiserum #3 against GST-PLZF improved its specificity since 1:1000 dilution detected GST-PLZF with the same sensitivity as an unpurified fraction, but detected GST-WT and GST to a much smaller extent (Figure 2A). I was able to eliminate the reactivity of #3 antiserum with GST-PLZF by pre-incubating it with GST-PLZF protein, but not by pre-incubation with GST, which demonstrates that a significant amount of the reactivity in #3 antisera is against the PLZF epitope (Fig.2B).

Analysis of the Monoclonal Mouse Antisera raised against PLZF. I screened several hybridoma supernatants by dot blot to select one with the strongest reaction to PLZF (Figure 3A). None of the five supernatants reacted with GSTor GST-WT. All of the hybridomas detected GST-PLZF, but none of them detected GST-9ZFPLZF. Out of five hybridomas we chose hybridoma 2A for our work.

Hybridoma 2A produces an IgG subtype 2A, as determined by Dr. T. Moran (Hybridoma core, Mount Sinai School of Medicine). I purified and concentrated the antibody using a protein A-agarose column from 500 mls of hybridoma supernatant and obtained 2.85 mg of antibodies (Table I).

Table I. Affinity purification of Hybridoma 2A9 supernatant.

Sample	O D	Concentration (mg/ml)	Volume	Protein (mg)	yield
end flow	0.30	8.10			
wash1	0.03	0.91			
wash2	0.02	0.84	50th ml		
wash 3	0.04	1.31	100th ml		
wash 4	0.01	0.42	150th ml		
wash 5	0.00	0.15	15 ml		
elution 1	0.00	0.15	1.5 ml		
elution2	0.00	0.15	1.5 ml		
elution3	0.00	0.15	1.5 ml		
elution4	0.04	**1.23**	1.5 ml	**1.8 mg**	
elution5	0.09	**2.56**	1.5 ml	**3.84 mg**	
			Total yield (mg)=	**5.64 mg**	
After dialysis	0.04	1.13	2.5 ml	**2.85 mg**	

Epitope mapping for hybridoma 2A was performed using whole cell extracts from CV1 cells expressing deletion mutants of PLZF. CV1 cells were transfected with SG5 plasmids (Stratagene) carrying GAL 1-147 DNA binding domain of GAL4 protein fused to various deletion mutants of PLZF

(plasmids were constructed by Jia Li and are described in Materials and methods). Western Blot analysis demonstrated that hybridoma 2A9 recognizes aa 100-200 of the PLZF protein (Figure3B).

DISCUSSION

We analyzed polyclonal rabbit and monoclonal mouse antibodies generated against PLZF. Polyclonal rabbit antiserum #3 had the highest specificity and sensitivity to GST-PLZF and also cross-reacted with GST epitope. Affinity purification of antiserum #3 improved its specificity for PLZF by decreasing its reactivity with GST and GST-WT. Mouse monoclonal antibodies secreted by hybridoma 2A recognized PLZF specifically and gave the strongest reaction. It specifically recognizes an epitope between amino acids 100-200 of PLZF.

Figure 1. Analysis of the rabbit polyclonal anti-PLZF sera. The indicated proteins were spotted onto Nitrocellulose membrane in the amounts ranging from 0.5 to 50 ng and blotted with three dilutions of each antisera.

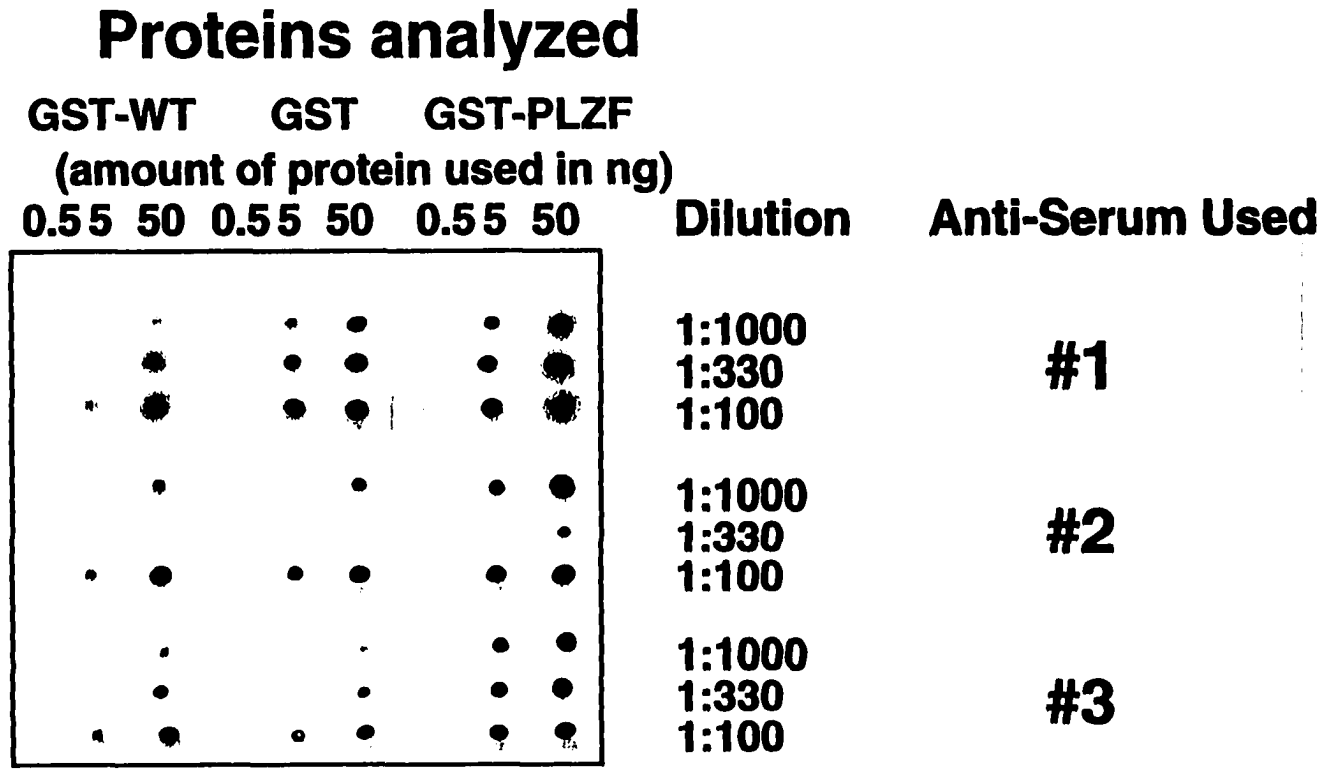


Figure 1. Analysis of the rabbit polyclonal anti PLZF sera. Various proteins were spotted onto Nitrocellulose membrane in the amounts ranging from 0.5 to 50 ng and blotted with three dilutions of each antisera.

Figure 2. Comparative analysis of antii PLZF serum #3, purified IgG and affinity purified antiserum. (A). GST fusion proteins were spotted onto Nitrocellulose membrane in indicated amounts and blotted with various fractions of antiserum #3. (B). Specificity of anti PLZF antibody . CV1 cells were transfected with 10 μg of PLZF expression vector. At 48 hours post-transfection whole cell extracts were electrophoretically separated and transferred to an Immobilion membrane. Membrane strips were blotted with 0.4 μg/ml of affinity purified anti-PLZF immunoglobulin. The antibody was preincubated with no competitor or a 100-fold molar excess of GST or GST-PLZF protein, as indicated.

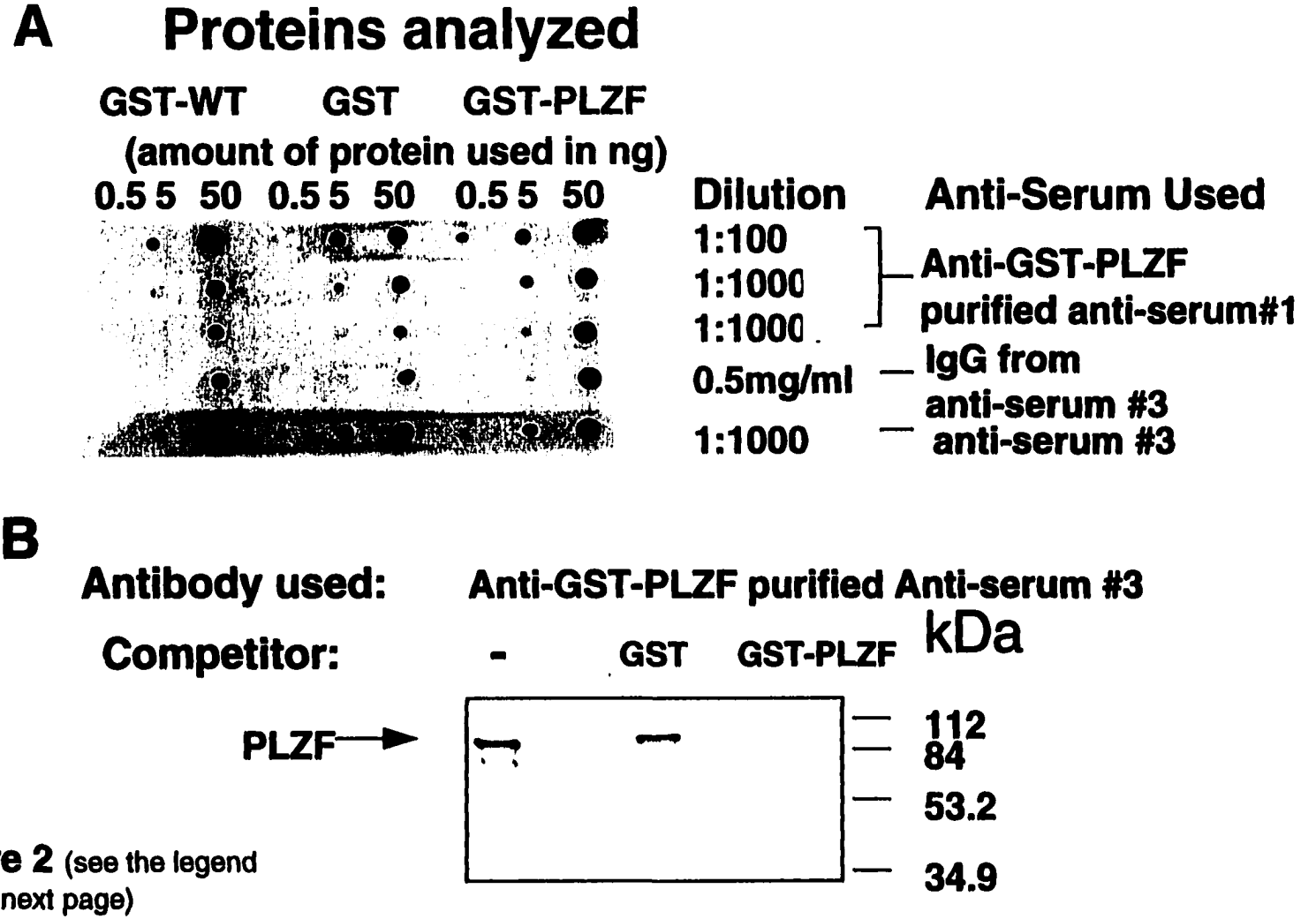


Figure 2 (see the legend on the next page)

Figure 3. Analysis of the mouse monoclonal hybridomas raised against PLZF. (A).Nitrocellulose membrane with GST fusion proteins spotted at concentration from 0.5 to 50 ng was blotted with mouse monoclonal hybridomas. (B). Whole cell extracts from CV1 cells transfected with plasmids coding for various deletion mutants of PLZF were separated on SDS-PAGE, electrophoretically transferred to PVDF membrane and blotted with I μg/ml of 2A monoclonal antibody.

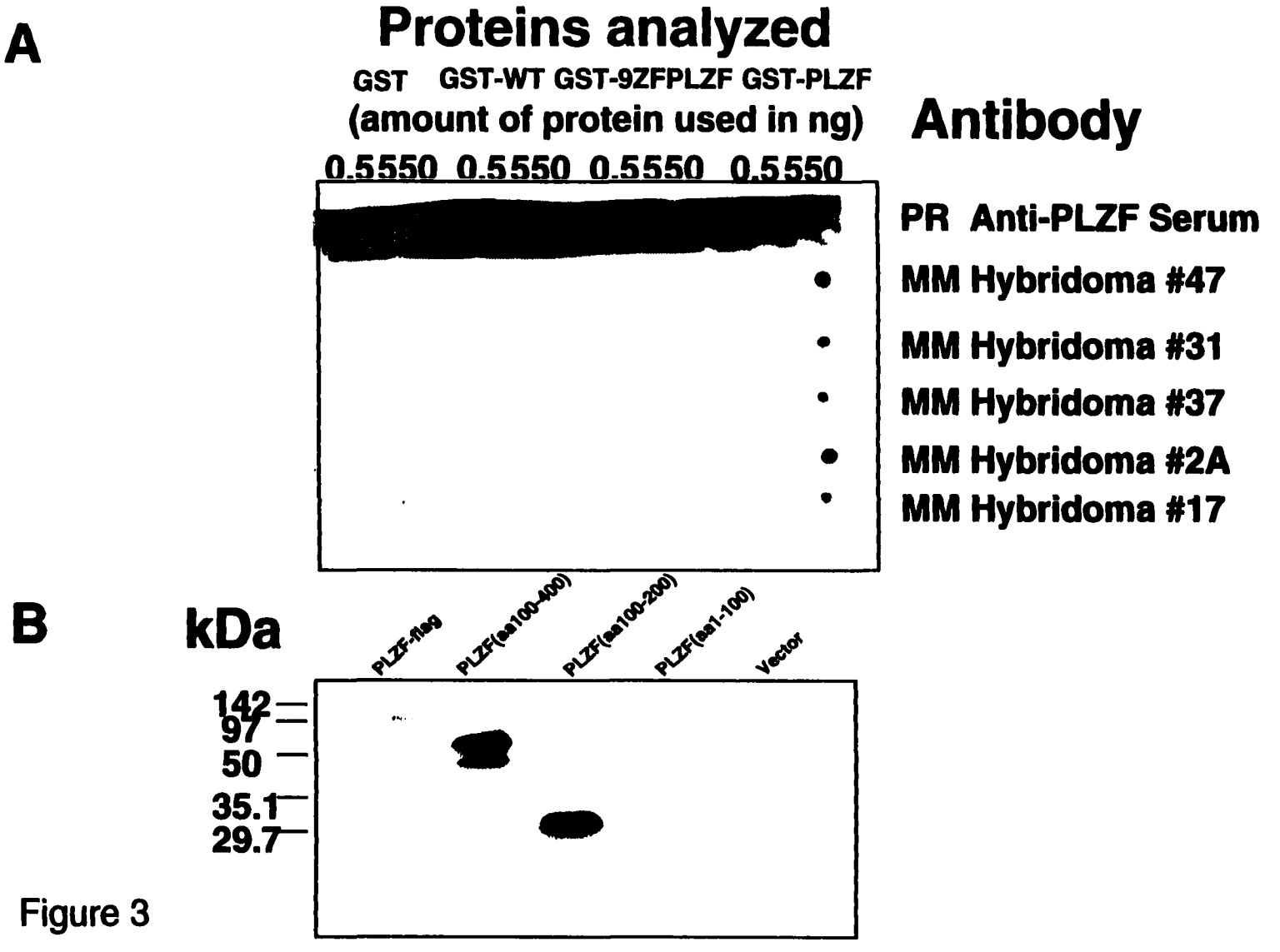


Figure 3

CHAPTER IV

Characterization of PLZF and its fusions with RAR

INTRODUCTION

The well characterized anti-PLZF antibodies (Chapter III) allowed us to visualize PLZF by the Western Blot and also to define its subcellular localization using immunofluorescent staining of the transfected cells. Using the antibodies against PLZF it became possible to study its expression in hematopoietic cells. We chose HL60 and U937 myeloid cell lines for the initial experiments since these are very well described sytems that are often used to study myeloid transcription factors. At the same time Dr. G. Acs (Biochemistry Depertment) found by Northen blot analysis (data not shown) that PLZF gene was highly expressed in a myelodysplastic cell line (MDS, established in his laboratory) (Banerjee et al., 1992) when treated with the calcium ionophore A23187. This cell line exhibits both T-cell and myeloid markers on its cell surface and upon induction with ionophore acquires a monocytic morphology (Banerjee et al., 1992). This cell line presented a system to study subcellular localization of PLZF and to investigate the possible co-localization with PML in PODs.

Our model for the development of t(11;17) APL included the possible novel functions that fusion proteins PLZF-RARα and RARα-PLZF could acquire in comparison with the wild type PLZF and RARα. I investigated the potential alternative subcellular localization and responsiveness to ATRA of the fusion proteins PLZF-RARα and RARα-PLZF.

RESULTS

The dynamic of PLZF expression in MDS cells. It was first observed that PLZF mRNA was upregulated after MDS cells were treated with Ca^{2+} ionophore A23187 (S.Weiland and Dr. G. Acs, unpublished results). The mechanism of action remains still unknown, possibly involving phospholipase C (PLC) or Ca^{2+} signalling. Having a tentative link between A23187 treatment and monocytic differentiation of MDS cells I researched the ability of several cytokines to induce PLZF upregulation in MDS cells (Figure 4A). None of the used factors produced the expected effect, suggesting the specificity of ionophore action. The cytokines were selected based on their contribution to differentiation along different myeloid pathways: monocytic (M-CSF) or granulocytic (GM-CSF) or their proliferative effect (IL-3). The lack of PLZF upregulation could reflect the unresponsiveness of MDS cells to tested cytokines or to differentiation conditions used.

A23187 induced PLZF expression in a concentration dependent manner. The lowest concentration tested that induced PLZF after 48 hr exposure was 100 nM (Figure 4B lane 10). 50nM A23187 did not induce detectable levels of PLZF (Figure 4B lane 11). Induction was stronger with 200 and

400 nM concentrations (Figure 4B lanes 8,9). We chose 400 nM concentration for the induction of MDS cells. The time necessary for PLZF induction in MDS cells was next studied (Figure 4C). It can be seen from the Figure 4C that it takes 24 hours of continuous exposure to induce PLZF expression (Figure 4C lane 12). Upregulation of PLZF expression by A23187 may be not a direct effect, since it takes between 4 and 24 hours. Optimization conditions for PLZF induction in MDS cells established a system in which the characterization of PLZF protein became possible.

Immunodetection of the PLZF protein in transfected CV1 cells, HL 60 and MDS cells. The cDNA sequence of PLZF predicts the production of a 673 amino acid protein with nine zinc finger domains. PLZF-flag was detected as a major 90 kD band in CV-1 cells transfected with the PLZF-flag expression vector (Figure 5A). The predicted molecular weight of the PLZF protein by conceptual translation of the PLZF cDNA is 74.2kd. Other zinc finger proteins such as Evi-1 have been found to migrate more slowly in SDS polyacrylamide gels than predicted (Matsugi et al., 1990). Several smaller and less intense bands of faster electrophoretic mobility were also noted probably representing partially proteolized species (Figure 5A). The specificity of recognition of PLZF-flag by the anti-PLZF antibody was confirmed by the fact that anti-flag antibody recognized a band of the same electrophoretic mobility.

We next examined the expression of PLZF protein in non-transfected hematopoietic cells. PLZF is expressed at a low RNA level in myeloid HL-60 cells (Chen et al., 1993) and immunoblots of these cells yielded a very

weak signal (Figure 5A). Upon induction of the MDS cells with a 48 hour treatment of 400 nM A23187 a 90 kD protein was detected in the MDS cells (Figure 5A). Together these data indicate that the PLZF gene is regulated in a hematopoietic cell and that the protein detected in these cells is of the same size as that expressed in transiently transfected CV1 cells. In addition only a single protein species was detected in the MDS cells. This suggests that the RNA processing event that generated the PLZF(A) protein, missing a 123 amino acid segment of the PLZF protein, is not occuring in the MDS cells.

Subcellular localization of PLZF in transfected CV1 cells, HL 60 and MDS cell lines. If PLZF encoded a transcription factor it might be expected to be localized to the nucleus at least some of the time. To test this possibility CV1 cells were transfected with SG5-PLZF expression vector (plasmids were described in Materials and Methods). Indirect immunofluorescence with polyclonal anti-PLZF antibody detected a nuclear protein, excluded from nucleoli (Figure 5B) in a fraction of the cells which had incorporated the PLZF expression vector. The pattern of nuclear staining was fairly uniform. Occasionally both nuclear and cytoplasmic staining was noted, perhaps due to overexpression of the PLZF protein and overload of the nuclear targeting apparatus. The PLZF localization in promyelocytic HL-60 cells was studied next and a faint speckled nuclear pattern was detected (Figure 5C). To better define this pattern, I performed immunofluorescent confocal microscopy in MDS cells induced with A23187, which according to the Western blot analysis express much higher

levels of PLZF protein. In treated cells PLZF protein was localizing to the numerous punctate domains somewhat reminiscent of PML-RARα domains in t(15;17) APL (Figure 5D). Although PLZF domains were more numerous, it was possible to say with some certainty that some of them co-localized with PML domains as seen by indirect immunostaining with polyclonal rabbit anti-PML and monoclonal mouse anti-PLZF antibodies, followed by secondary anti-mouse and anti-rabbit antibodies conjugated to FITC and Texas Red respectively. Two-channel confocal microscopy gave a picture of PML co-localizing with PLZF, but controls showed that the secondary anti-mouse antibody was cross-reacting with anti-PML rabbit polyclonal serum and made the results uninterpretable. The attempts to eliminate cross-reactivity were unsuccessful and the patterns of separate PML and PLZF staining in A23187 treated MDS cells were compared. Laser scanning confocal microscopy was performed using a Leica system (Leica, Germany).

PLZF is a phosphoprotein. Gene regulation by transcription factors in response to various stimuli does not necessarily require novel protein synthesis. Many transcription factors are regulated by post-translational modifications, for example by phosphorylation. The PLZF protein has several Ser, Thr and Tyr amino acids in its sequence, that are potential targets for phosphorylation. I wanted to test whether these amino acids could be phosphorylated *in vivo*. CV1 cells were transfected with 10 μg of the PLZF-Flag expression vector or a *c-fos* expression vector (Lloyd et al., 1991). Monoclonal anti-fos antibody, but not the Flag monoclonal antibodies immunoprecipitated the ~52 kd FOS protein which was detected in both methionine and phosphate labeled extracts (Figure 6 lanes 4, 8). In

parallel, the anti-Flag M2 antibody, which recognizes the Flag epitope when present internal to a protein, but not the M1 antibody, which only recognized the Flag epitope when present on the N-terminus of a protein immunoprecipitated a 90 kD PLZF phospoprotein (Figure 6 lanes 2,6). PLZF therefore is phosphorylated when expressed in eukaryotic cells, although not as extensively as the FOS protein (Figure 6, compare lanes 2 and 6 vs 4 and 8). PLZF protein can be phosphorylated *in vitro* by cyclin A dependent kinases (Dr. Zelent, unpublished data), suggesting that PLZF could play a role in the control of the cell cycle. In order to determine which amino acids are phosphorylated *in vivo* CV1 cells transfected with the PLZF-flag vector were labeled with [^{32}P] as described above. I immunoprecipitated the labeled protein, performed SDS-PAGE and transferred the protein band to PVDF-immobilion membrane. The 90 kD ^{32}P labeled protein band was cut out, the protein eluted and subjected to trypsin digestion and acid hydrolysis by Dr. R.Kohanski in the Protein Core facility of the Mount Sinai Hospital. The mixture of single amino acids and incompletely digested peptides were electrophoretically separated on a TLC plate in two dimensions at pH1.9 and pH3.5 respectively and compared to standards (Cooper et al., 1983) The analysis showed that PLZF is phosphorylated on Ser and Thr (phosphoaminoacid analysis was performed by Dr. R.Kohanski).

Expression of PLZF in myeloid compartment can not be detected by the Western Blot. PLZF expression can be detected by immunostaining in CD34+ bone marrow cells and by RT-PCR in hematopoietic 32D cells, FDCPMixA4 cells and in NB4 and HL 60 cells

before differentiation with RA (Chen et al., 1993; Reid et al., 1995). Western Blot was used to study specific expression of PLZF in the myeloid compartment (Figure7). As I showed in Figure 5C low levels of PLZF expression can be seen in HL 60 cells by immunofluorescent microscopy technique. Western Blot could not detect PLZF expression in HL 60 and U937 cell lines before or after differentiation with RA. Nor could PLZF be detected in peripheral blood macrophages or a macrophage cell line G2 (Figure 7).

Western Blot did not prove to be a useful technique to study PLZF expression in various cell lines. It seems that levels of PLZF expression in those cell lines are relatively low and can be detected by a sensitive RT-PCR or RNA-protection technique, but not by the Western Blot.

Subcellular localization of PLZF-RARα and RARα-PLZF.

The localization of the fusion proteins is important for understanding the mechanism of APL associated with t(11;17). We wanted to compare the localization of the PLZF fusion proteins to the PML-RARα fusion, that was shown to localize to the cytoplasm in the absence of RA, and to translocate to the nucleus, when cells were treated with pharmacological doses of ATRA (Kastner et al., 1992). This translocation is believed to underly the success of differentiation therapy in patients with t(15;17). PLZF(A)-RARα and PLZF(B)-RARα localize to the nucleus in the presence and in the absence of treatment with 10^{-6} M ATRA (Figure 8A). No apperent change in subcellular pattern of distribution could be detected with conventional microscopy, although it is possible that fine changes are taking place in the distribution of the PLZF-RARα fusions. In order to visualize fusion proteins

by the Western Blot CV1 cells were transfected with PLZF(A)-RARα, PLZF(B)-RARα or RARα-PLZF in pSG5 expression vectors containing SV40 promoter/enhancer (Stratagene). PLZF(A)-RARα and PLZF(B)-RARα were detected as stable protein species of 90 and 105 kD respectively, also higher than the predicted molecular weights of 81 kD and 93 kD (Fig. 3B).

It was more difficult to obtain information about the small RARα-PLZF fusion protein, because of the lack of the suitable antibody. Anti-PLZF antibody did not recognize the carboxy-terminal fingers of the PLZF (as demonstrated in Chapter III), and anti-RARα (A1) antibody was not effective in immunofluorescenct staining. In order to circumvent this problem we decided to resort to fractionation of transfected CV1 cells. Nuclear and cytoplasmic extracts were immunoblotted with anti-RAR(A1) antibody (a gift of Dr. P.Chambon). The localization of RARα-PLZF fusion in this experiment was mostly nuclear and was not effected by ATRA treatment.

DISCUSSION

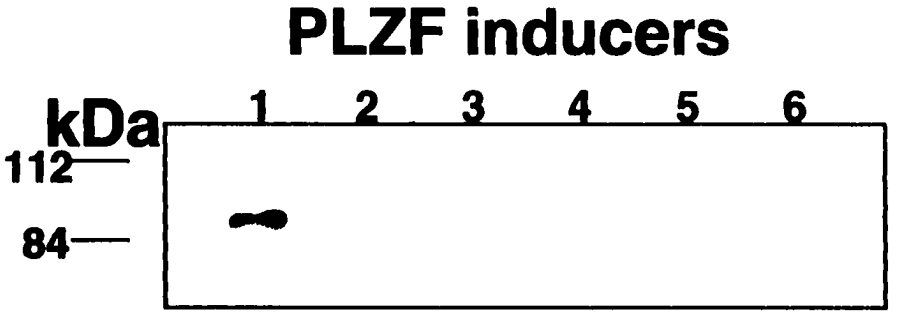
Nuclear localization and phosphorylation of PLZF on Serines and Threonines correspond well to its putative role as a transcription factor. The significance of phosphorylation is not yet clear. It could be of great importance in regulating the function of PLZF as a transcription factor, particularly in the light of the fact that PLZF may be regulating the cyclin A gene and post-translational modification may be a fast way to regulate the

regulator thoughout the cell cycle. Phosphorylation was shown to effect protein activity by allosteric conformational changes and by changes in electrostatic properties (for an overview, see (Hunter and Pines, 1994)). For example, DNA binding by transcription factors like c-fos, max and myogenin is inhibited after phosphorylation of certain amino acids. Phosphorylation of GAL4 and Sp1 was correlated with transcriptional activation. In transfected CV-1 cells PLZF yields diffuse nuclear staining, but in HL-60 and A23187 treated MDS cells the localization is in distinct nuclear speckles of unknown nature. Similarly PML, when expressed transiently gave a variaty of staining patterns including cytoplasmic and diffuse nuclear (Daniel et al., 1993; Kastner et al., 1992; Koken et al., 1994). An abnormal non-physiological distribution of protein in transient over-expression systems could be inevitable consequence of over-production and saturation of the protein processing and targeting apparatus. In MDS cells, PLZF speckles were more numerous and mostly separate from the PML POD domains. Since simultaneous staining against PML and PLZF in MDS cells was not possible due to antibody cross-reactivity, it is impossible to be certain that none of PLZF localizes to PODs, even though it is certain that at least some of PLZF localized to distinct domains. We do not know whether PLZF localization in APL patients is effected because the blood samples of t(11;17) patients are rare and not obtainable.

Localization of PLZF(A) and (B)-RARα proteins is also nuclear and is not effected by ATRA treatment, suggesting that PLZF-RARα does not function through aberrant localization. On the other hand, t(15;17) is associated with aberrant microspeckled localization of PML-RARα and PML in NB4 and APL cells, that is restored to the wild type PODs after ATRA treatment (Dyck et al., 1995; Weis et al., 1994). At the same time, the RARα-PLZF is mostly a nuclear protein with a small detectable fraction in the cytoplasm. The cytoplasmic localization of RARα-PLZF could be the result of the limitations of the transfection system, in which a protein can be overproduced and not targeted properly, and also of the crudeness of cell fractination by hypotonic swelling. Based on our transient transfection studies localization of PLZF-RARα and RARα-PLZF can not explain the aberrant function predicted for the chimeric proteins, but the potential effect of translocation on localization can not be ruled out until the studies can be done in APL cells with t(11;17), which are not available due to the low number of patients.

Figure 4. Dynamics of PLZF expression in MDS cells. (A) MDS were cultivated in the presence of various agents: 400nM A23187, 10u/ml M-CSF, 60 ng/ml GM-CSF, 5 u/ml IL-3, Interferon γ. (B) MDS cells were treated with various concentratons of A23187 for 48 hours. (C) MDS cells were treated with 400 nM A23187 for the lengths of time indicated in the figure. After treatment whole cell extracts were made, electrophoretically separated on SDS-PAGE and transferred to Immobilion membrane. The membrane was blotted with anti-PLZF serum at a 1:1000 dilution.

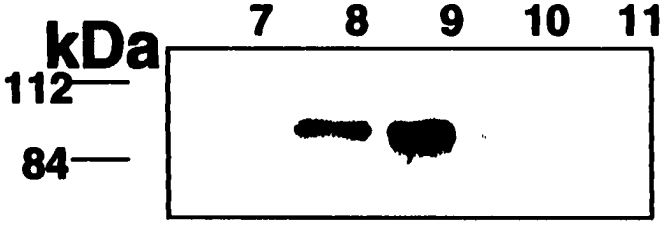
A



1. A23187 400 nM
2. Control
3. M-CSF
4. GM-CSF
5. IL-3
6. Interferon γ

What concentrations of A23187 induce PLZF?

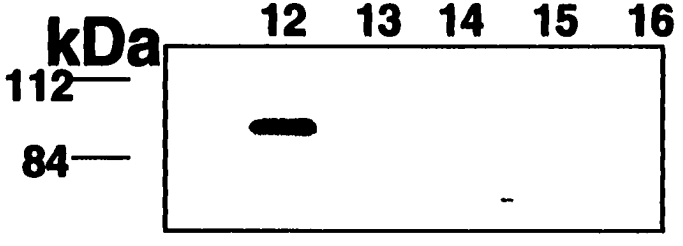
B



7. Control
8. 400 nM
9. 200 nM
10. 100 nM
11. 50 nM

Time necessary to induce PLZF with 400nM A23187

C



12. 24 hs
13. 4 hs
14. 30 min
15. 10 min
16. control

Figure 4

Figure 5. Expression of PLZF in transfected CV1 cells, HL 60 and myelodysplastic cells. (A) Specificity of PLZF antibodies. CV1 cells were transfected with 10 μg of PLZF-Flag expression vector. At 48 hours post-transfection whole cell extracts were made. Whole cell extracts were also made from HL 60 cells and MDS cells that were treated with 400 nM A23187 for 48 hours. Extracts were electrophoretically separated, transferred to Imobilion PVDF membrane and immunoblotted with polyclonal anti-PLZF serum or preimmune serum. (B) CV1 cells were transfected with 10 μg of PLZF-Flag, expression vector. Nuclear localization of Flag epitope labeled PLZF was detected with polyclonal rabbit anti-PLZF antibody and a 1:2000 dilution of fluorescene-conjugated mouse anti-rabbit IgG. (C) Cytospins of HL-60 cells were stained with rabbit polyclonal PLZF antisera (1:1000) and detected with fluorescene-conjugated goat anti-rabbit IgG (1:2000). (D) MDS cells were grown with 400 nM A23187 for 48 hours, immobilized on polylysine coated slides, immunostained and analyzed by confocal microscopy. PLZF protein was detected by 1:1000 dilution of rabbit polyclonal anti-PLZF antibody, PML protein was detected by 1:500 dilution of polyclonal anti-PML antibody (Gift of Dr. Redner). Control polyclonal rabbit anti-mouse antibody was used at 1:500 dilution.

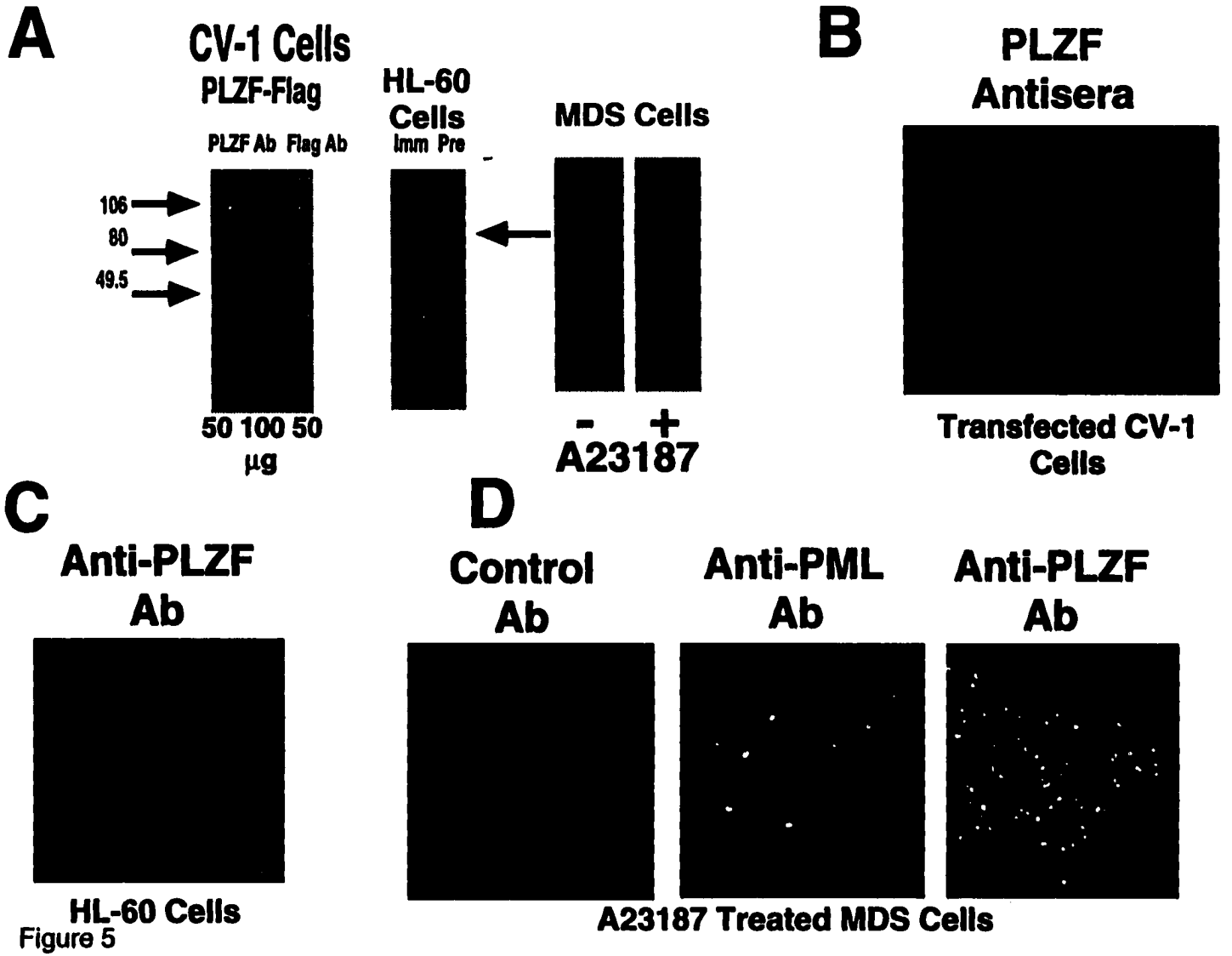


Figure 5

Figure 6. PLZF is phosphorylated when expressed in CV1 cells. CV1 cells, transfected with 10 mg of PLZF-Flag or *c-fos* expression plasmid were labeled with [^{32}P] orthophosphate or [^{35}S] methionine. Extracts from PLZF-Flag transfected cells were subjected to immunoprecipitation using 3 μg of anti-Flag M2 or M1 monoclonal antibody. Proteins from *c-fos* transfected cells were immunoprecipitated using 1 μg of anti-fos monoclonal antibody (Santa Cruz Immunologics) or 1 μg of M1 monoclonal antibody. Immunoprecipitated proteins were separated through a 10% SDS polyacrylamide gel and visualized by fluorography or autoradiography.

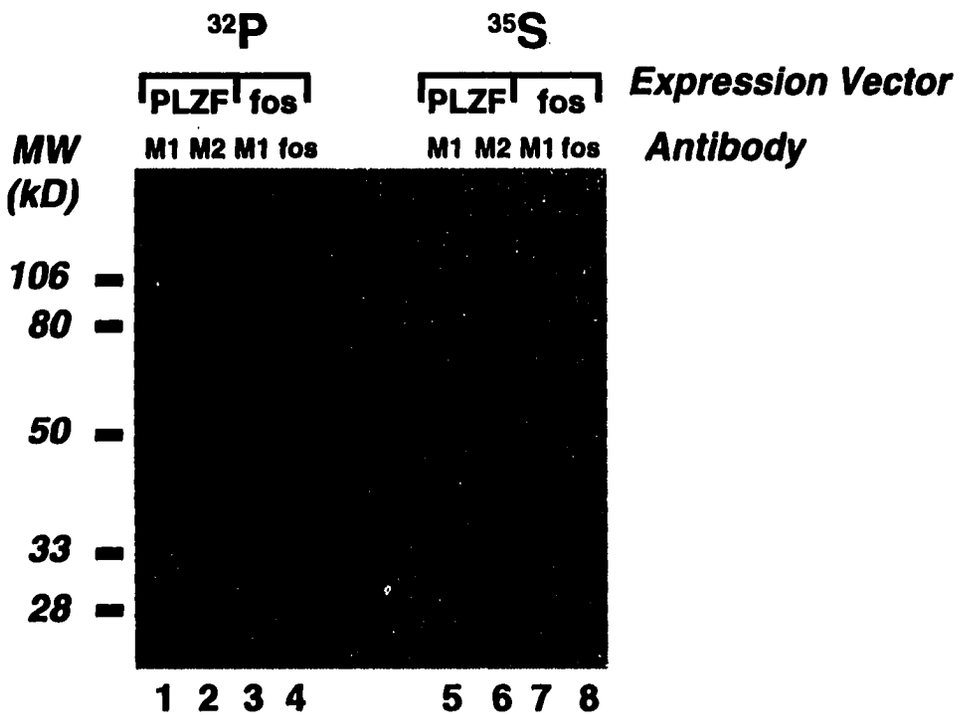
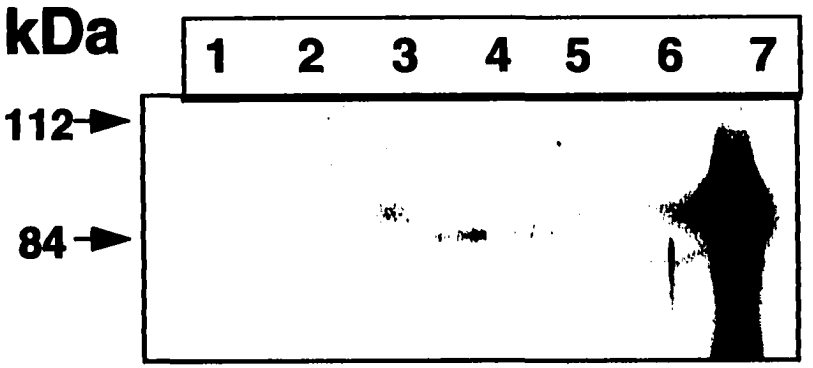


Figure 6

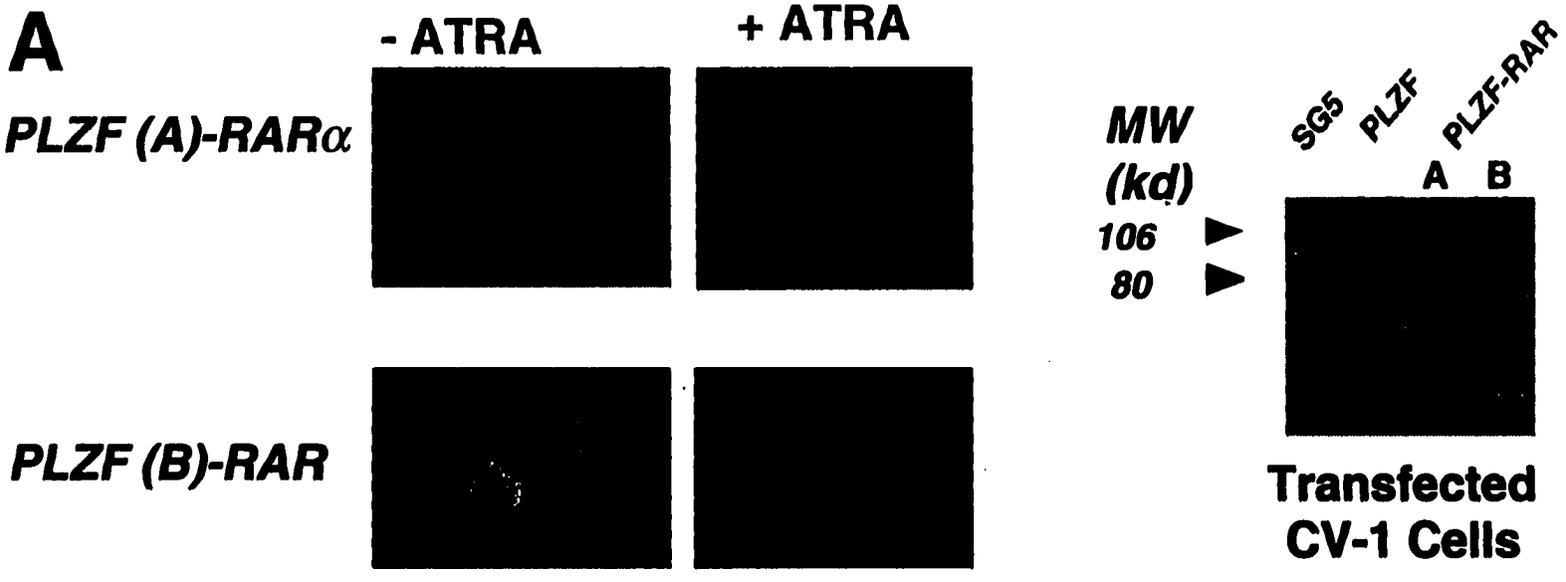
Figure 7. Cell type specific expression of PLZF is undetectable by the Western Blot. Whole cell extracts were made from the indicated cell lines. Where specified, cells were grown in the presence of differentiating agent 10^{-6} M all-*trans* retinoic acid or its ethanol carrier. Extracts were electrophoretically transferred to PVDF membrane and blotted with a 1:1000 dilution of rabbit polyclonal anti-PLZF serum. *In vitro* transcribed and translated PLZF-FLAG was used as a positive control.



1. U937 +RA
2. U937 + Carrier
3. HL 60 +RA
4. HL 60 + Carrier
5. Peripheral Macrophages
6. G2 cell line
7. TNT PLZF-FLAG

Figure 7

Figure 8. Cellular localization of wild type PLZF, PLZF-RARα and RARα-PLZF fusion proteins. (A) CV1 cells were transfected with 10 μg of PLZF(A)-RARα or PLZF(B)-RARα and grown in the absence and in the presence of 10^{-6} M all-*trans* retinoic acid. PLZF proteins were detected with polyclonal PLZF antiserum (1:1000) and fluorescene-conjugated goat anti rabbit IgG (1:2000). (B) CV-1 cells were transfected with 10 mg of SG5, PLZF or PLZF-RARα plasmids and after 48 hours the whole cell extracts were electrophoretically separated. Proteins were transferred to PVDF membrane and blotted with 1:1000 dilution of polyclonal PLZF antiserum (C) Nuclear and cytoplasmic extracts from CV1 cells transfected with 10 μg of RARα1-PLZF expression plasmid, were separated through a 15% SDS polyacrylamide gel and transferred to Immobilion PVDF membrane. Protein was detected with a 1:500 dilution of monoclonal anti-RARα1 antibody .



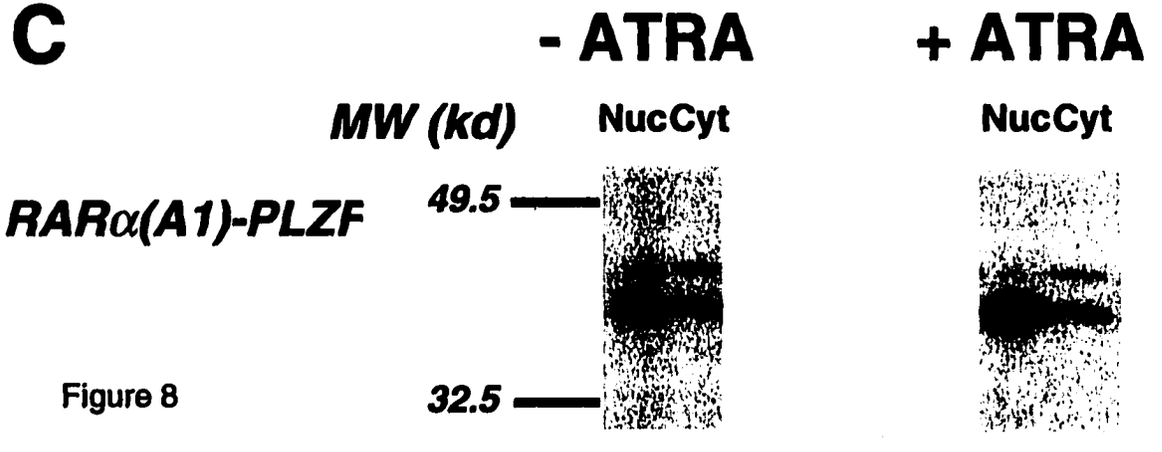


Figure 8

CHAPTER V

Generation of myeloid cells stably expressing PLZF

INTRODUCTION

In order to study the role that PLZF plays in differentiation of myeloid lineage I decided to overexpress it in a myeloid cell line. Forced expression is used to study the function of many hematopoietic factors, for example Early growth response-1 (EGR-1) protein. Northern blot analysis with poly(A)+ RNA from HL 60 cells showed that EGR-1 is not expressed in undifferentiated or DMSO-induced granulocyte-like HL 60 cells, but is induced with transient kinetics in PMA-induced macrophage-like HL 60 cells (46). Egr-1 antisense oligomers blocked macrophage differentiation of HL 60 cells in the presence of macrophage inducers PMA or PMA and DMSO. At the same time, Nguen *et al.* demonstrated that overexpression of Egr-1 in stably transformed HL60 cells committed them towards monocytic, but not granulocytic lineage (46). Thus, it appears that EGR-1 is

a transcription factor which is required for monocytic differentiation and prevents granulocytic differentiation in myeloid cells. On the other hand, overexpression of Helix-Loop-Helix (HLH) protein Id inhibited granulocytic differentiation of 32DCl3(G) cells treated with G-CSF. Downregulation of Id is necessary to release basic-HLH proteins involved in execution of specific differentiation programs and to allow them to bind to E-box DNA motif (Kreider et al., 1992).

To study the effect PLZF produces on myeloid differentiation I wanted to use human myeloid cell line HL-60 or U 937 and to express PLZF under an inducible promoter. There are several inducible promoters available for expression of factors in the mammalian system. Widely used ones include the metallothioneine promoter, tetracycline-controlled transactivator (tTA)-responsive promoter (Tet system) (Paulus et al., 1996) and ecdysone-inducible expression promoter (Invitrogen, San Diego, Ca). I cloned PLZF and its fusions with RAR into pSVneoHMTIIa-Ter plasmid that contains human metallothioneine promoter and allows induction of cDNA transcription upon addition of Zn^{+2} into the growth medium. An inducible system allows to overexpress factors that may have detrimental effects on the cell growth by allowing to select stable clones first and then to upregulate expression of the protein of interest. pSVneoPLZF was transiently transfected into CV1 cells, but expression of PLZF protein could

not be upregulated (Figure 9). After that I constructed EboplppPLZF vector that allowed upregulation of PLZF expression through amplification of episomal vector after increasing hygromycin concentration in the growth medium. This system had a partial success with phenotypic effect of PLZF overexpression, but PLZF upregulation was difficult to detect and cell pools did not survive long enough to study the effect on differentiation. I finally switched to the use of a retroviral vector, pBabepuro, and the retroviral packaging cell line Ψ2 as a quick and efficient system for transducing a cell line of interest, even though it provides not inducible expression of the studied protein. The pBabepuro retroviral vector expresses cDNA from the Mo MuLV Long Terminal Repeat (LTR) and transmits cloned cDNA at high titres.

We chose mouse the myeloid 32DG/GM cell line as a model of myeloid differentiation. 32DG/GM is a murine interleukin-3 dependent myeloid cell line that was obtained from normal mouse bone marrow, has a diploid karyotype and does not form tumors in syngeneic mice (Greenberger et al., 1983). It differentiates into neutrophilic granulocytes after exposure to granulocyte colony-stimulating factor (G-CSF) and into macrophages after exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF) (Kreider et al., 1990). The 32DG/GM cell line is a popular model of hematopoietic development and has been used to study the effects of oncogenes (Anderson et al., 1990; Mavilio et al., 1989; Rovera et al., 1987),

tumor suppressor genes (Soddu et al., 1996) and growth factors (Shimada et al., 1993).

RESULTS

Failure to upregulate PLZF expression from pSVneoHMTIIa-PLZF plasmid. In order to create a cell culture system with inducible PLZF expression I cloned PLZF into pSVneoHMTIIa-Ter vector containing human metallothioneine promoter. Addition of 100 μM ZnCl2 induces metallothioneine promoter and thus should upregulate the expression of the inserted cDNA. To test the pSVneoHMTIIa-PLZF construct I transiently transfected CV1 cells and 24 hours after transfection added 100 μM ZnCl2 for 7 or 25 hours. The efficiency of transfection was high as judged by Growth Hormone measurements (see Materials and Methods). Immunoblotting of whole cell extracts of the transfected and induced cells demonstrates that PLZF expression is not upregulated after addition of an inducing agent (Figure 9). There is a very faint band in lanes 2,3 and 4 that co-migrates with PLZF-flag control in lane 1. Since PLZF is probably not expressed in CV1 cells (my immunostaining and immunoblotting data, Figure 8 A and B) the faint band may be a non-specific background band or may represent a true and specific low level of PLZF expression in CV1

cells. In any case there is no induction of PLZF expression from pSVneoHMTIIa-PLZF plasmid. Not being able to express PLZF from the pSVneoHMTIIa-Ter vector in the transient system I decided not to use it for creation of the stably expressing clones and to switch to a different inducible system.

Stable NB4D5 clones containing EboplppPLZF sense and EboplppPLZF antisense plasmids. I next decided to use episomal based inducible system for stable overexpression of PLZF. Eboplpp vector contains SV40 promoter and hygromycin B resistence gene. When it is incorporated into the cell its numbers can be amplified by increasing concentrations of Hygromycin B. Our colleague Dr. Ethan Dmitrovsky successfully used this system in NB4D5 acute promyelocytic cell line carrying translocation t(15;17) and highly responsive to differentiation with ATRA for expression of RAR and Pml (Ahn et al., 1995). I constructed Eboplpp PLZF vector with PLZF cDNA in the sense and antisense orientations and Dr. Dmitrovsky used these vectors to electroporate NB4D5 cells according to the established in his laboratory protocol. The initial analysis was done in the laboratory of Dr. Waxman and demonstrated by the western blot the ability to upregulate PLZF expression after raising hygromycin concentration. The cell lines seemed to be growth suppressed and were lost before they could be expanded and cryopreserved. After new stable clones were generated they were handed to me for analysis of PLZF expression and phenotype. The western blot analysis of the whole

cell extracts demonstrated that PLZF was not expressed at an easily detectable levels even after hygromycin concentration was raised to 200μg/ml (Figure 10A). There is a faint 90 kD band in lanes 3, 4 and 5 that co-migrates with TNT produced PLZF and demonstrates low levels of endogenous expression of PLZF in NB4D5 cells. The level is not upregulated in Ebopipp PLZF Sense clones (Figure 10A lanes2,4,6). Extract from 10^6 live cells was loaded in each lane of the gel in Figure 10A, but lanes 1,2 and 6 seem to contain less protein and have less "background" probably due to the proteolysis as a result of the increased cell death in the lines cultured in the presence of 200 μg/ml of hygromycin.

Even though the levels of PLZF expression were very low and could not be upregulated in stable NB4D5 clones, I compared the growth rates of clones carrying sense and antisense PLZF cDNA (Figure 10 B and C). Even in the absence of hygromycin NB4D5 PLZF Sense seemed to grow slightly slower after plating (smaller slope of the growth curve in Figure 10B) and reach lower density than Antisense clone. In the presence of 65μg/ml of hygromycin NB4D5 PLZF Sense was growth suppressed reaching the saturation density of $5x10^5$, while PLZF Antisense clone reached a density of $1.7x10^6$ (Figure 10C). At 200μg/ml of hygromycin both sense and antisense clones were growth suppressed perhaps due to hygromycin

effect with greater growth retardation in the Sense clone (Figure 10C). All cells were dead by day 7 of cultivation in the presence of the drug. NB4D5 clones could not be maintained in culture for prolonged periods of time and hygromycin concentrations necessary for detectable upregulation of PLZF produced significant cytotoxicity. These reasons in addition to the fact that NB4 cells contain t(15;17) and PML-RARα and therefore is not a pure system to study the function of PLZF prompted me to look for a different system to overexpress PLZF.

Creation of ψ2 clones packaging PLZF and transduction of 32DG/GM cell line. ψ2 cells were electroporated with pBabepuroPLZF or insertless pBabepuro and stably transfected cells were selected in puromycin and cylinder cloned. PLZF expression was verified by immunoblotting of whole cell extracts from ψ2 clones. From five clones tested in Figure 11 three express PLZF (lanes 3, 4 and 5). I confirmed the ability of PLZF expressing clones to package infection-competent retrovirus by the ability of supernatant to confer puromycin resistance to infected 3T3 cells.

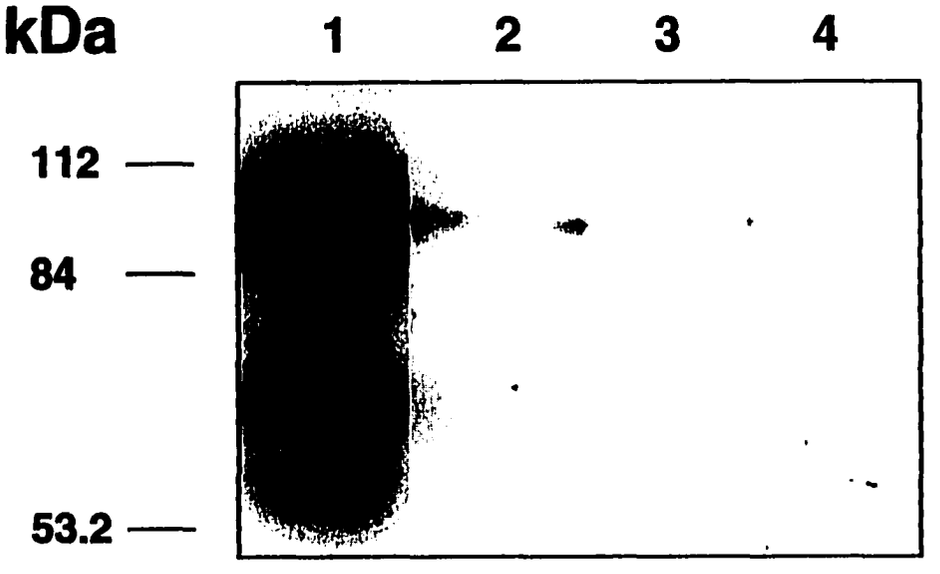
Supernatant was collected from ψ2 PLZF-2 and 3 clones and used to infect 32DG/GM cells overnight. Puromycin resistant 32DG/GM pools were selected for 2-3 weeks and then tested for expression of PLZF. PLZF expression in the stable pools was demonstrated by immunoblotting (Figure 12). Six 32DG/GM PLZF pools analyzed all expressed a similar level of PLZF, that was much greater than the endogenous expression in 32DG/GM cells, where PLZF mRNA is detectable by RT-PCR (Reid et al., 1995), but the protein is not detected by the Western Blot (Figure 12).

DISCUSSION

My attempts to create an inducible PLZF cell line were not successful. The faint bands observed on the Western blots of induced and uninduced cells carrying pSVneoHMTIIa-PLZF probably represent low levels of PLZF expression from metallothioneine promoter that has basal activity in the absence of added Zn^{2+} (Figure 9). Similar low levels of basal expression were observed with Ebopipp plasmid in NB4D5 cells (Figure 10A). It is possible that inability to upregulate PLZF expression was due to the growth inhibitory effect PLZF had on the chosen cell lines and that the low levels of PLZF expression were the only levels tolerated in those cell lines. (See Results in Chapter VI). Growth suppressive effect of PLZF could be seen even from the growth curves of transfected NB4D5 clones. Even in the

absence of hygromycin the Sense clone grew slower than the Antisense clone and the difference in growth rates increased after addition of 65μg/ml of hygromycin. This is consistent with growth suppressive effect of PLZF even though its upregulation could not be detected by the western blot in NB4D5 cells. Even in the presence of 200μg/ml of hygromycin, which produced general cytotoxic effect suppressing growth of all the clones, Antisense clone grew slower and to the lower density. The growth suppressive effect of PLZF and inability to maintain stable expression of PLZF for a long time was corroborated by other researchers. Dr. A. Zelent observed inhibition of cell growth by PLZF in BALF3 cell line that was later lost and Dr. Z. Chen observed a similar effect of PLZF overexpression in 32DCL3 cells that also could not be maintained in cell culture (personal communications). Hematopoietic cell lines appear to be more sensitive to PLZF effect, since PLZF produces only very mild effect on the growth rate of 3T3 and ψ2 cell lines (unpublished data from Dr. P. Yeyati). I was able to create stable ψ2 clones packaging PLZF containing retrovirus and to transduce 32DG/GM cells with it. It is not clear why 32DG/GM cells can tolerate higher levels of PLZF expression.

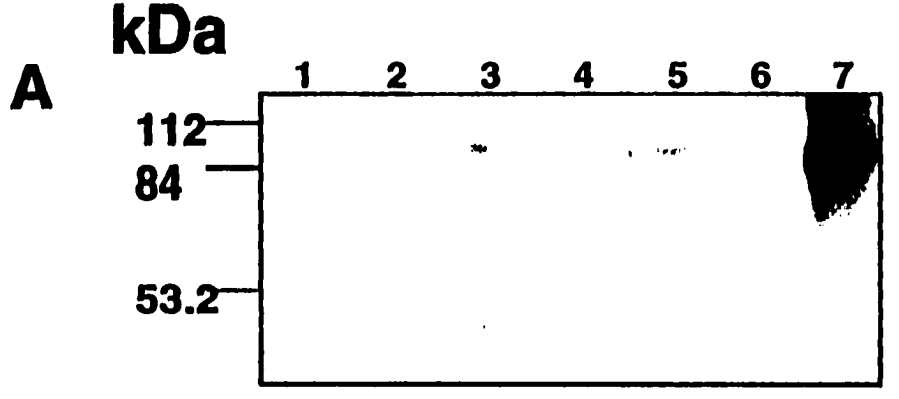
Figure 9. PLZF expression is not induced from pSVneoHMTIIa-PLZF plasmid. CV1 cells were transiently transfected with pSG5PLZF-flag or pSVneoHMTIIa-PLZF and cells were treated with 100 μM $ZnCl_2$ for 7 to 25 hours as indicated in the figure to induce PLZF expression. After this cells were collected, whole cell extracts were electrophoretically separated and transferred to Immobilion membrane. The membrane was blotted with affinity purified 2A9 antibody at 1μg/ml.



1.PLZF-flag
2.MT-PLZF+Zn 7 hs
3.MT-PLZFuninduced
4.MT-PLZF+Zn 25 hs

Figure 9

Figure 10. Stable NB4D5 clones with inducible episomal PLZF expression system. (A). Stable NB4D5 clones carrying PLZF cDNA in the Sense or Antisense orientation were treated with 65 or 200 μg/ml of Hygromycin for the number of days indicated in the figure. Whole cell extracts were resolved through 7.5% SDS polyacrylamide gel and transferred to Immobilion PVDF membrane. The membrane was blotted with affinity purified 2A9 antibody. (B). NB4D5 clones were plated at $1x10^5$ cells/ml and live cells were counted on the indicated days using trypan blue exclusion. (C). NB4D5 clones were plated at 2x105 cells/ml in the presence of hygromycin at the indicated concentration. Live cells were counted on days 2, 4, 7 above.



Cell line	Hygromycin Concentration	# Days
1.Antisense	200	4
2.Sense	200	4
3.Antisense	65	4
4.Sense	65	4
5.Antisense	200	2
6.Sense	200	2
7.TNT PLZF		

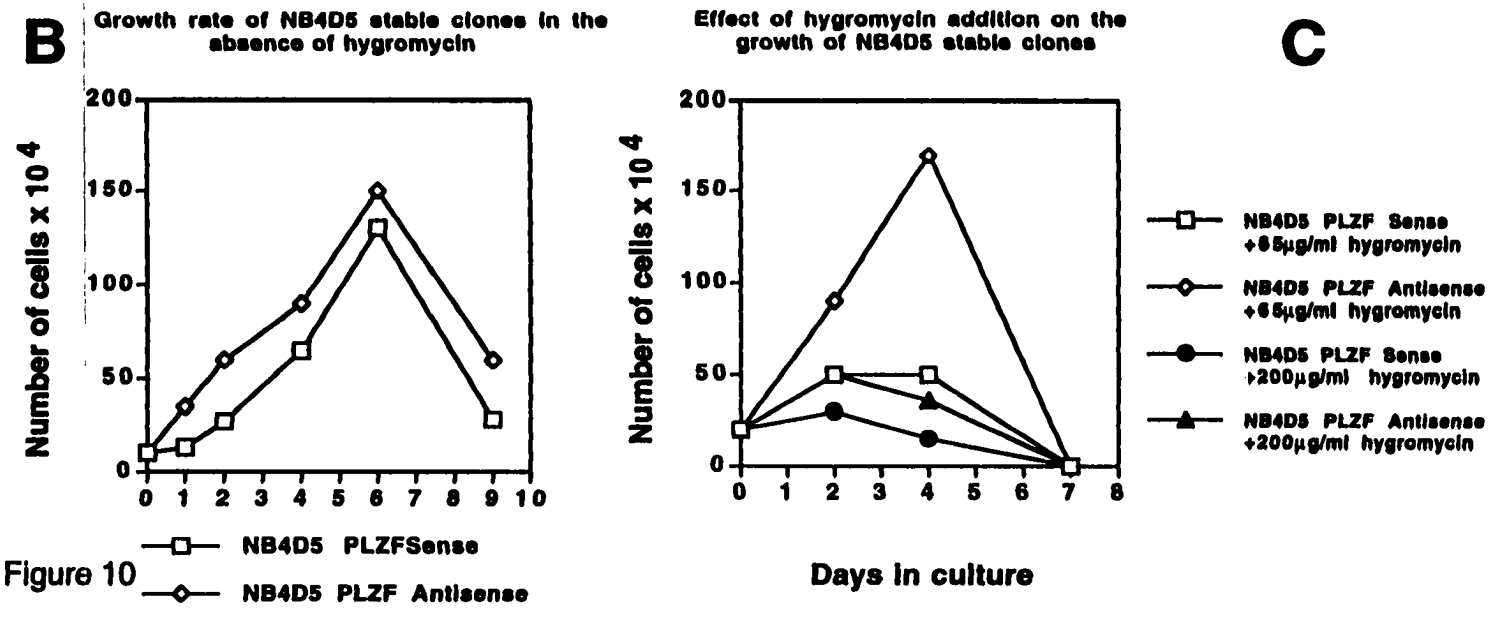


Figure 10

Figure 11. Expression of PLZF in the stable clones of ψ2 retroviral packaging cell line. Whole cell extracts made from $1x10^6$ cells from the indicated ψ2 clones were separated on SDS PAGE and immunoblotted with affinity purified mouse anti-PLZF antibody as described above.

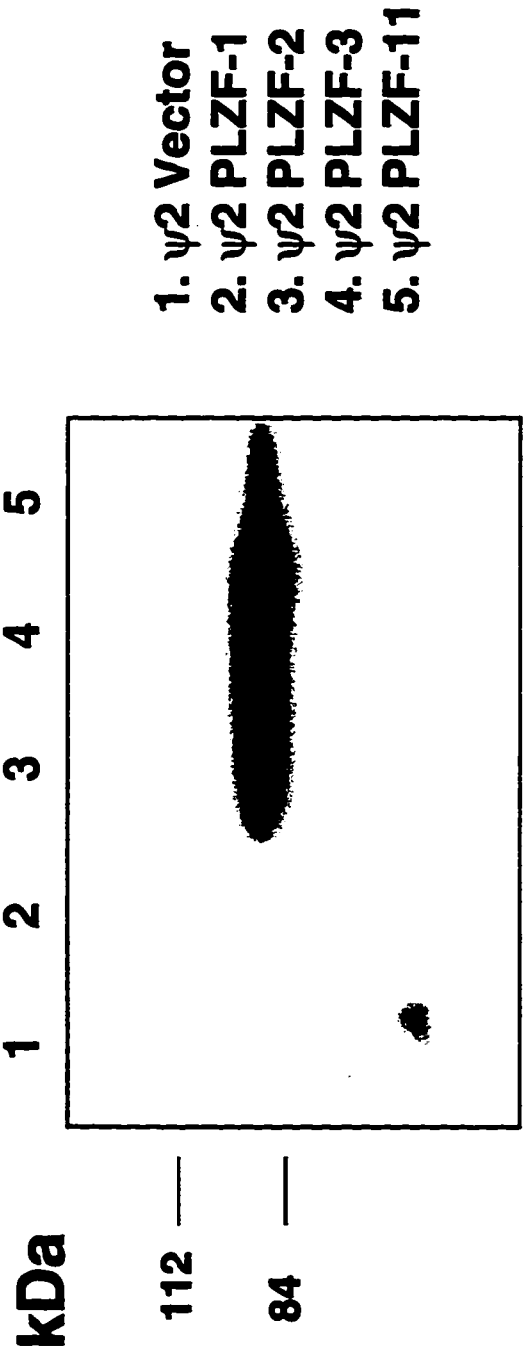


Figure 11

Figure 12. Analysis of retrovirally-infected 32DG/GM pools for exogenous expression of PLZF. In vitro translated PLZF and whole cell lysates from 32DG/GM Vector and PLZF pools were separated by electrophoresis through a denaturing 10% polyacrylamide gel, transfered to PVDF membrane and blotted with monoclonal mouse anti-PLZF antibody 2A9.

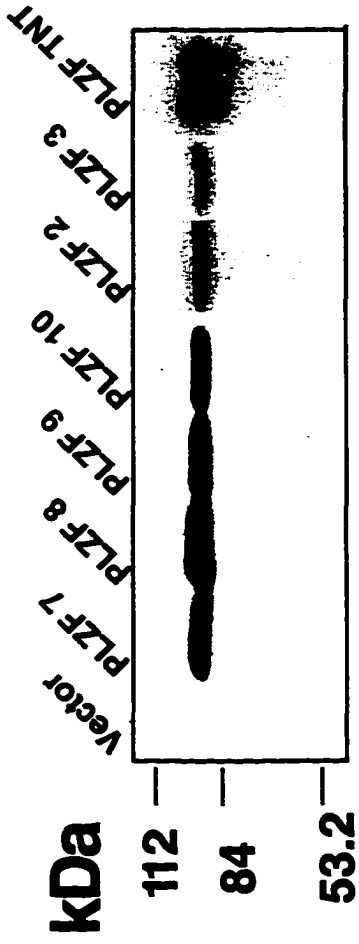


Figure 12

CHAPTER VI

Overexpression of PLZF in 32DG/GM cell line causes growth suppression, G1 arrest and increase in apoptosis.

INTRODUCTION

The 32DG/GM cell line is a subclone of 32DCL3(G) cell line established from mouse bone marrow by Greenberger *et al.* (Greenberger et al., 1983). The 32Dcl3 cell line was originally responsive to IL-3 and G-CSF, but after priming with G-CSF it upregulated GM-CSF receptors and became competent to respond to both cytokines with differentiation along granulocytic or monocytic lineages (Kreider et al., 1990). Therefore the 32DG/GM cell line is a convenient hematopoietic system to study monocytic and granulocytic development.

Based on the published experiments we chose 32DG/GM cell line as a model to study the effect PLZF would produce on myeloid differentiation. No target genes for PLZF were known at that time and we could not derive PLZF function by homology with other known proteins. The system that

provided an insight for our work was t(15;17) APL and extensive research of the PML function. Even though the structure of PML and PLZF are very dissimilar they may have some similarities of function. Both of these proteins may contribute to the control of cell proliferation and differentiation, which is why their translocation may be associated with the development of leukemia. PML-RARα was shown to exert a dominant-negative transcriptional effect over RARα (de The et al., 1991; Kakizuka et al., 1991; Kastner et al., 1992). When PML-RARα was overexpressed in HL 60 and U937 cell lines it inhibited myeloid differentiation, suggesting its key role in development of differentiation block. PML and RARα are implicated in the control of proliferation since their overexpression in NB4 cells caused significant growth suppression (Ahn et al., 1995). Overexpression of PML also suppressed growth of NB4 cells on soft agar and their tumorigenicity in nude mice as well as transformation of NIH 3T3 cells by activated *neu* oncogene (Mu et al., 1994). The difficulty of overexpressing PLZF in NB4 and 32DG/GM cell lines together with an observed growth suppression of NB4 cells expressing the PLZF sense cDNA suggested to us that PLZF may also suppress cell growth when overexpressed and may be directly involved in the control of the cell cycle.

RESULTS

PLZF expression in 32DG/GM cells is associated with growth suppression and G1 arrest. PLZF expressing pools of 32DG/GM cells grew extremely slowly (Figure 13). Wild type and vector alone cells had a very similar growth curves and reached plateau of growth at about day 5 at a density exceeding 10^6 cells/ml, while PLZF pools were severely growth retarded and never reached a density greater than 10^5 cells/ml. PLZF 2 pool could be maintained in culture for a very limited time. The doubling time for wild type and vector cell lines was less than 24 hours, in contrast it took PLZF pools on average more than 3 days to double their population.

In order to understand the nature of the growth retardation we studied the cell cycle profile of PLZF expressing 32DG/GM pools. The propidium iodide staining of DNA demonstrated no difference in the cell cycle profile of cells infected with insertless retrovirus and wild type cells, which contained less than 50% of cells in G0/G1 compartment (Figure 14A). At the same time the profiles of PLZF overexpressing pools were significantly altered with up to 80% of cells in G0/G1. This distortion of the cell cycle could reflect the prolonged G1 phase or a complete block of some cells in G1. The histograms also showed an increased sub-G0/G1 shoulder, indicative of the cell death.

Most of the cells expressing PLZF are blocked in G0/G1, while the minority of the cells cycle normally (Figure 15). It was shown before that withdrawal of IL-3 from the growing 32Dcl3 cells results in G1 arrest and apoptosis (Blandino et al., 1995). Vector and PLZF 2 pools were plated in Conditioned Medium (CM) without IL-3 and after 48 hours cells were stained with PI and cell cycle distribution was analysed using FACS. At the start of the experiment PLZF 2 pool had 65% of cells in G1, while the Vector pool had 50% of cells in G1. After 48 hours of growth without IL-3 PLZF 2 pool contained 74% of total still living cells in G1 (Figure 15). At the same time the Vector pool had most of the cells in a subG1 peak with approximately 10% of cells in the G1 phase of the cell cycle.

Reversal of growth suppression is associated with PLZF downregulation. A direct confirmatioin of the PLZF induced phenotype is that the phenotype is completely or partially lost after downregulation of the PLZF expression. When PLZF 7 and 10 pools displayed faster growth and loss of GO/G1 block, they also demonstrated the loss of PLZF expression by the Western Blot (Figure 15B and C). PLZF was selectively downregulated, while the pool still maintained the puromycin resistanse.

PLZF overexpression leads to apoptosis. The growth suppression of PLZF expressing pools could be explained by slower proliferation of the cells or by their accelerated death. In the case of PLZF overexpression it seems that both mechanisms are true. PLZF expressing pools of 32DG/GM cells had higher percentage of the cells in the sub G1 peak, that is consistent with DNA degradation in cells undergoing apoptosis (Figure 16A). To demonstrate apoptosis more definitively we used the TUNEL assay to detect free 3'OH DNA ends (Figure 16B). 32DG/GM cells infected with empty retroviral vector grown in IL-3 had 5% of cells undergoing apoptosis, which is likely a reflection of the physiologic turnover of cells. The PLZF2 pool had up to 11% of cells positive for apoptosis by TUNEL Assay: a two fold increase relative to the vector pool (Figure 16B).

Apoptosis was also demonstrated by Annexin V staining of the phosphoserines on the outer surface of the dying cell. PLZF expressing 32DG/GM pools on average had 2-3 fold more cells undergoing apoptosis (Figure 16C) than vector pools. The percentage of cells in subG1 peak and the result of other assays for apoptosis correlate with each other and consistently demonstrate increased cell death in the PLZF-expressing pools. A direct link between expression of PLZF and apoptosis was demonstrated by dual labelling of the cells. Two-color FACS analysis was

performed by staining cells for PLZF using anti-PLZF antibody and for apoptosis using the TUNEL staining. PLZF staining was detected on FL2 by anti-mouse PE secondary antibody, while biotin-conjugated nucleotides, labelling free 3'-OH ends of the DNA, were detected on FL1 using avidin-FITC. From Figure 17A it was calculated that 44% of PLZF 2 pool cells stained positively for PLZF (shift to the right from the isotype control in black falling under the M1 marker). As can be seen from Figure 17B there are 4.71% of cells positive for apoptosis (right upper and lower quadrants of Figure 17B). Out of 4.71% of apoptosis positive cells 4.58% also express PLZF and fall to the upper right quadrant. Double staining allowed to correlate PLZF expression and apoptosis.

The overexpression of wt-p53 accelerates apoptosis of 32D cells after IL-3 withdrawal and G1 accumulation (Blandino et al., 1995). In the PLZF2 pool, we observed G1 accumulation and increased apoptosis, so we decided to determine if p53 protein was upregulated in these cells. However, 32DG/GM cells expressed the wild type p53 at the level undetectable by the western blot. The western blot did not detect p53 expression in PLZF pool (Figure 18). It is possible that the levels of p53 were below detectable by this method.

IL-3 receptor is downregulated in PLZF pools. 32DG/GM cells depend on IL-3 for survival and self-renewal. Downregulation of the IL-3 receptor could be perceived by cells as a paucity of the cytokine and could lead to G1 arrest and apoptosis. We pursued this idea by examining IL-3 receptor expression in the control and PLZF pools. We used an antibody that specifically recognizes the α subunit of the murine IL-3 receptor as a 70 kDa protein. The result of the Western Blot demonstrated that PLZF2 pool expresses a lower level of IL-3 receptor as compared to the vector pool (Figure 19A). In addition the mobility of the IL-3 receptor was faster in PLZF 2 cells, probably reflecting an absence of the usual glycosylation. To support this result we performed flow cytometric analysis of the cells stained with the same antibody against IL-3 receptor. Vector and a wild type pools had two to three times more IL-3 receptor positive cells than the PLZF pools (Figure 19B). The western blot result for PLZF 2 pool was confirmed by the FACS result demonstrating three fold downregulation of the IL 3 receptor.

DISCUSSION

Overexpression of PLZF in 32DG/GM cells produced dramatic growth suppression and block in G1 phase of the cell cycle. Since PLZF is highly

expressed in CD34+ cells and in cells that have not undergone terminal differentiation PLZF may be a regulatory factor that maintains hematopoietic precursors in unproliferative state. Stem cells are dormant in the G0 phase of the cell cycle and have limited proliferative capacity (Leary et al., 1992; Ogawa, 1993) Early hematopoietic progenitors may remain dormant for up to two weeks. Overexpression of PLZF significantly delays cells in G0/G1, making their cell cycle as long as 72 hours. The majority of PLZF expressing cells are blocked in G1, so that even after 48 hours of IL-3 withdrawal, when most of Vector cells undergo apoptosis, 74% of live PLZF cells remain in G1. The pools escape cell death and maintain PLZF expression. One possible explanation is that the block in G0/G1 phase is not complete and cells retain self-renewing capacity, even though they loose the proliferative capacity of a partially differentiated myeloblasts. Limited cell proliferation can be seen from the growth curves. The PLZF pools do not significantly increase the cell numbers, but rather reach a plateau density and maintain low cell numbers (Figure 13). Cell cycle profiles of PLZF pools demonstrated increased number of cells in G1 compartment and decreased number of cells in S and G2/M Figure 14). There is strong evidence that PLZF may cause cell cycle arrest by downregulating cyclin A expression (P.Yeyati *et al.*., in preparation), which is necessary for entering into S phase of the cell cycle. My preliminary data suggest that PLZF may also downregulate RB protein expression and phosphorylation (data not shown). It was demonstrated by Hatzfeld *et al.*

(Hatzfeld et al., 1991) that RB is necessary to maintain multipotential progenitors in quiescence, since antisense RB oligos released these cells from the block. PLZF may be affecting RB and thus causing the growth arrest.

The results also demonstrate that decreased proliferative capacity in PLZF overexpressing pools may be connected to increased rate of death by apoptosis. The results from Annexin staining and TUNEL assay correlate well with DNA analysis and percentages of cells in subG1 phase of the cell cycle. 32DG/GM PLZF pools 7 and 9 have highest Annexin V staining of 15 and 20 % respectively which constitutes most of the cells in subG1 peak equal to 20% in PLZF7 and 22% in PLZF9 (Figure 16). The double staining experiment in Figure 17 prooved the causal relationship between PLZF expression and apoptosis, since most of apoptosis was detected in PLZF positive cells. As far as I could detect increased apoptosis was not associated with upregulation of p53 expression and may not be due to a p53 dependent pathway. Overexpression of wt-p53 does not effect differentiation and morphology of 32D cells, but accelerates apoptosis when 32D cells are deprived of IL-3 (Blandino et al., 1995). Only 44% of cells in 32DG/GM PLZF2 pool were PLZF positive even though all cells in the pool were puromycin resistant. It is possible that expression of PLZF in the given cell fluctuates becoming undetectable by FACS. It is also

possible that a subset of the pool has no PLZF expression due to insertion of the retrovirus in a transcriptionally inactive portion of chromosome.

I also propose that PLZF effects proliferation of the stable pools by down-regulating the expression of IL-3 receptor. IL-3 synergizes with Steel factor, LIF and IL-6 to maintain proliferation and survival of CD 34^+ progenitors (Leary et al., 1992; Ogawa, 1993). More importantlly, 32DG/GM is an IL-3 dependent cell line and it arrests in G1 and undergoes apoptosis when withdrawn from IL-3. PLZF decreases IL-3 receptor expression two to three fold possibly causing lower proliferative capacity in the cell pools. There is some evidence that IL-3 Receptor density can control leukemic cell growth. In the study by Budel *et al.* cells from most cases of AML (13 out of 15) demonstrated high affinity IL-3 receptors (Budel et al., 1989). One case that did not proliferate in response to IL 3 demonstrated no IL-3 receptors. There is also evidence that overexpression of G-CSF receptors leads to increased proliferative response in 32Dcl3 cells. When the product of t(8;21) AML1-MTG8 was expressed in 32Dcl3 cells it resulted in upregulation of G-CSF receptor and increased proliferation without terminal differentiation (Shimizu et al., 1997). All of these data on expression of IL-3 receptors employed assays indirectly measuring IL-3 receptors by using labelled IL-3 ligand. It was suggested that the number of IL-3 receptors on the surface of the

hematopoetic cells can be from 20 to 300 per cell. I could detect receptors only on a subset of cells probably due to the low levels of receptor expression and limited resolution of the technique. Even though the numbers probably do not represent the true physiological situation, I think they are useful for the purpose of comparing one cell line to the other. There are multiple pathways controlling progression of blast cells through the cell cycle and IL-3 signal may provide proliferative competence (Budel et al., 1989). It was demonstrated by multiple studies that IL-3 produces proliferative signal by JAK2 activation or phosphorylation of Shc and Grb2 that signal through MAPK. If the normal function of PLZF is to repress IL-3 receptor expression, directly or indirectly, then RARα-PLZF, which has part of the RAR activation domain instead of two repression domains of PLZF, might activate IL-3 receptor expression and thus amplify the response of APL cells to the ambient cytokine. Since we studied only IL-3 Receptor α subunit, unique to IL-3 receptor, we do not know whether β subunit, which is shared by IL-3, IL-5 and GM-CSF receptors is effected by PLZF. If it were, PLZF would not only have an effect on proliferation, but also an effect on myeloid differentiation. The role of PLZF in the control of proliferation and apoptosis is particularly significant in the light of the fact that PLZF is rearranged in APL.

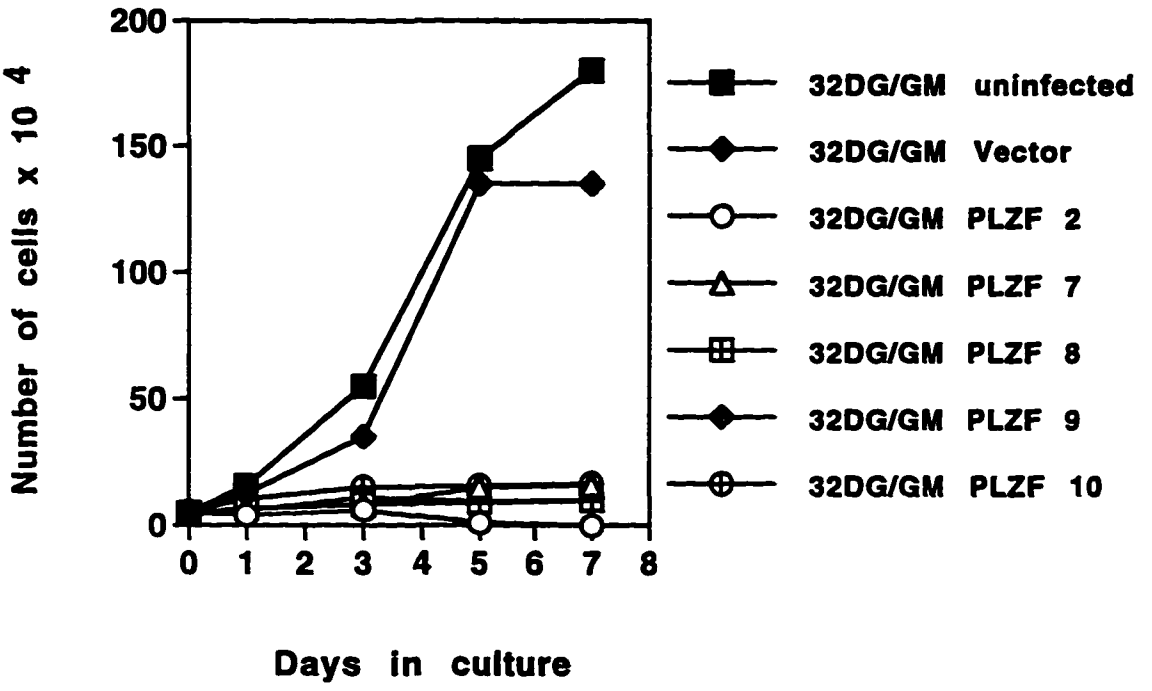


Figure 13. Effect of PLZF overexpression on cell proliferation. Duplicate cultures were plated at 5x104 cells per ml in complete medium. Live cell number was determined in duplicate, at two day intervals, with a Thomas's hemocytometer using trypan blue exclusion.

Figure 14. Effect of PLZF expression on cell cycle profile of 32DG/GM cells. (A). Cell cycle distribution of DNA content, as defined by PI staining, between G1/G0, S and G2/M phases of the cell cycle is displayed. (B). Cells grown in IL-3 were permeabilized with 0.2% Tween and DNA was stained with 10μg/ml of PI. Selected DNA histograms are shown.

A

Cell line	G_0/G_1	S	G_2/M
Uninfected	47	37	16
Vector	45	38	17
PLZF2	69	10	21
PLZF 7	67	22	10
PLZF8	59	25	16
PLZF 9	68	22	10
PLZF 10	80	9	11

B

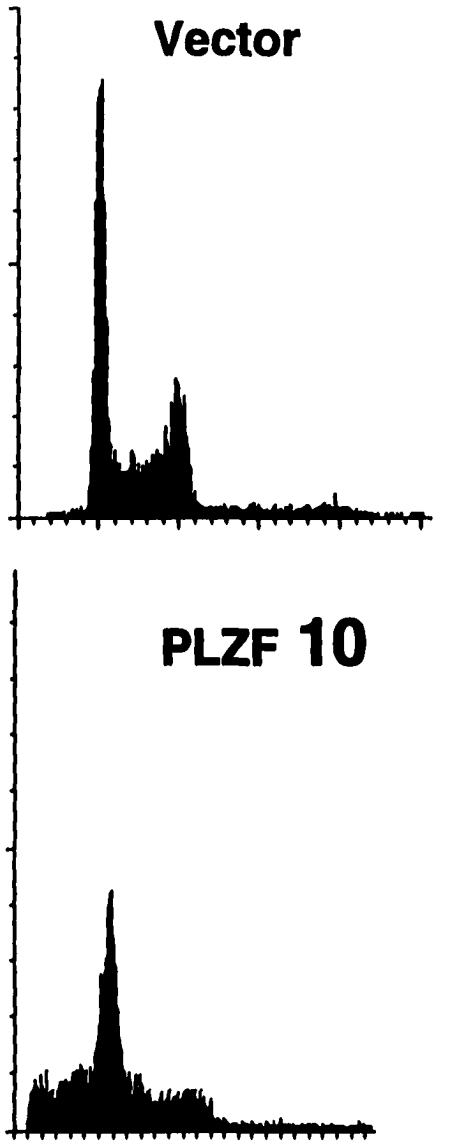


Figure 14

Figure 15 (A). PLZF expressing cells are blocked in G1 phase of the cell cycle. Vector and PLZF 2 pools were grown in the absence of IL-3 for 48 hours after which cells were collected and stained with PI. DNA content was analysed by FACS. Statistical analysis of the DNA content is indicated next to the histogram.

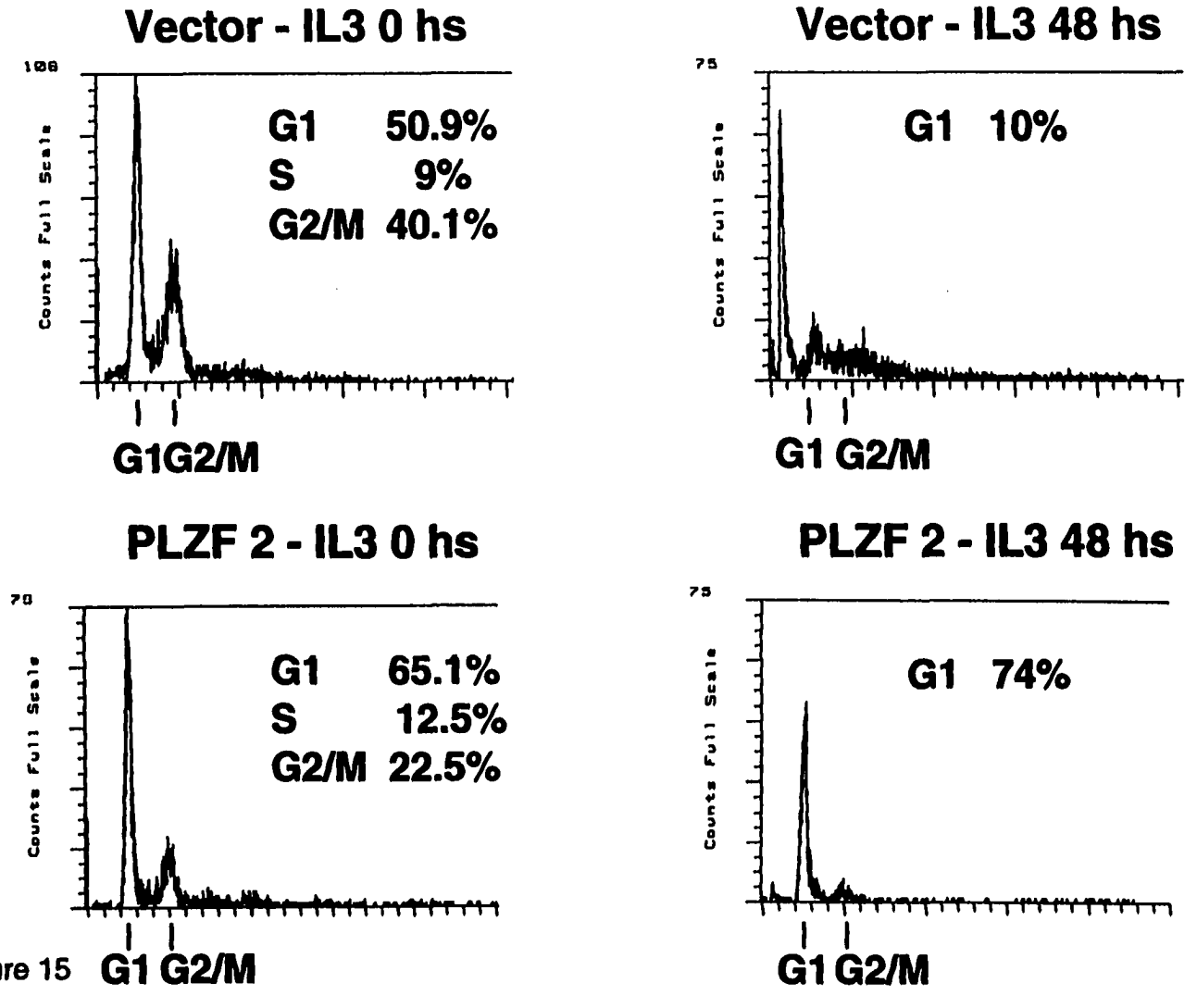


Figure 15

Figure 15 (B,C). The loss of PLZF induced phenotype is associated with downregulation of PLZF expression in the pools. (B). Whole cell extracts from Vector pool, PLZF 7 pool and two reverted pools PLZF 8R and PLZF 10R were separated by SDS-PAGE, transfered to PVDF membrane and blotted with anti-PLZF antibody. (C). Assessment of growth rate was performed by plating cells at $1x10^5$ per ml in complete medium and counting trypan blue excluding cells on the indicated days.

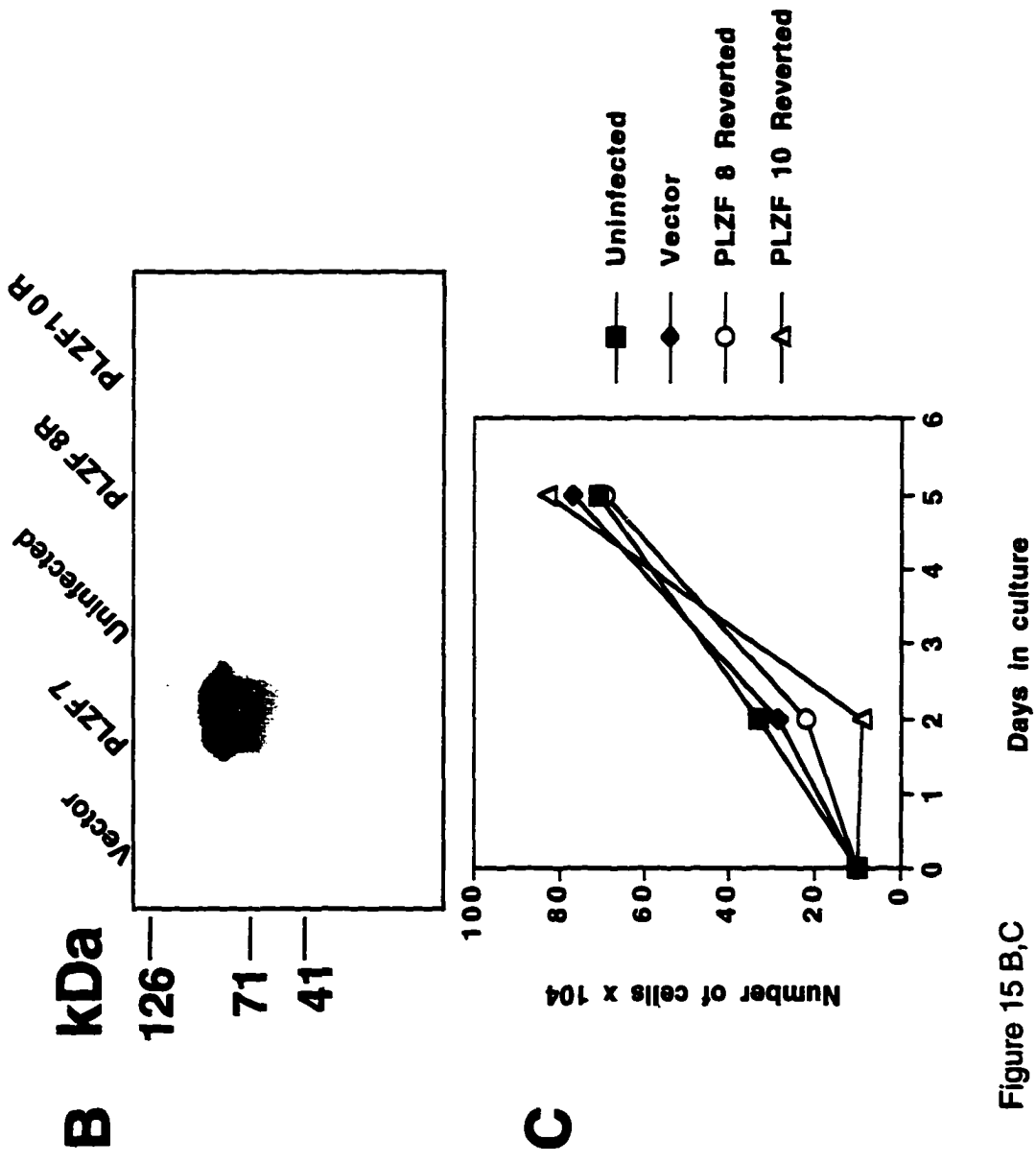


Figure 15 B,C

Figure 16. Effect of PLZF on programmed cell death in 32DG/GM cells grown in IL-3. (A).The percent of cells in subG1 fraction of the cell cycle was derived by integration of the histograms of DNA content. (B). Apoptotic cells were detected by modified TUNEL Assay (Oncor). The increase in apoptosis was derived by substracting the percentage of apoptotic cells in the Vector pool from the percentage of apoptotic cells in PLZF pools. (Vector pool had up to 5% of apoptotic cells.) (C). Live cells were stained with FITC-conjugated Annexin V. Necrotic cells were eliminated from the analysis by gating out all PI positive cells. (Vector pool had up to 10% of apoptotic cells by this assay). The increase in apoptosis was derived as above.

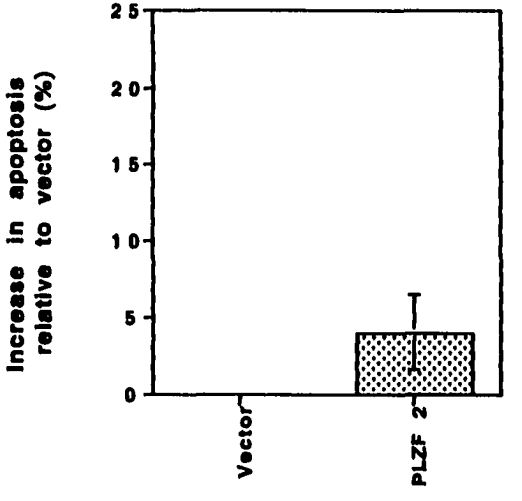
A

% sub G1 cells

Uninfected	<1%
Vector	<1%
PLZF 2	7%
PLZF7	20%
PLZF8	6%
PLZF9	22%
PLZF10	16%

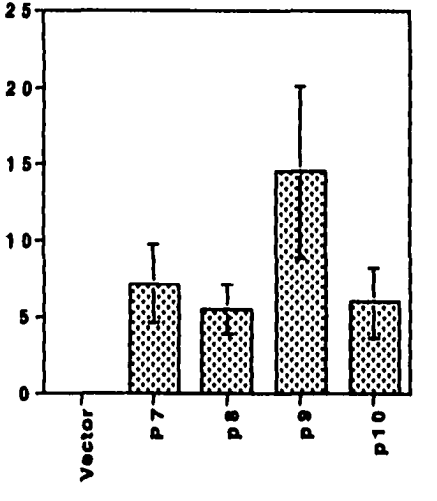
B

TUNEL Assay



C

Annexin V



Cell line

Figure 16

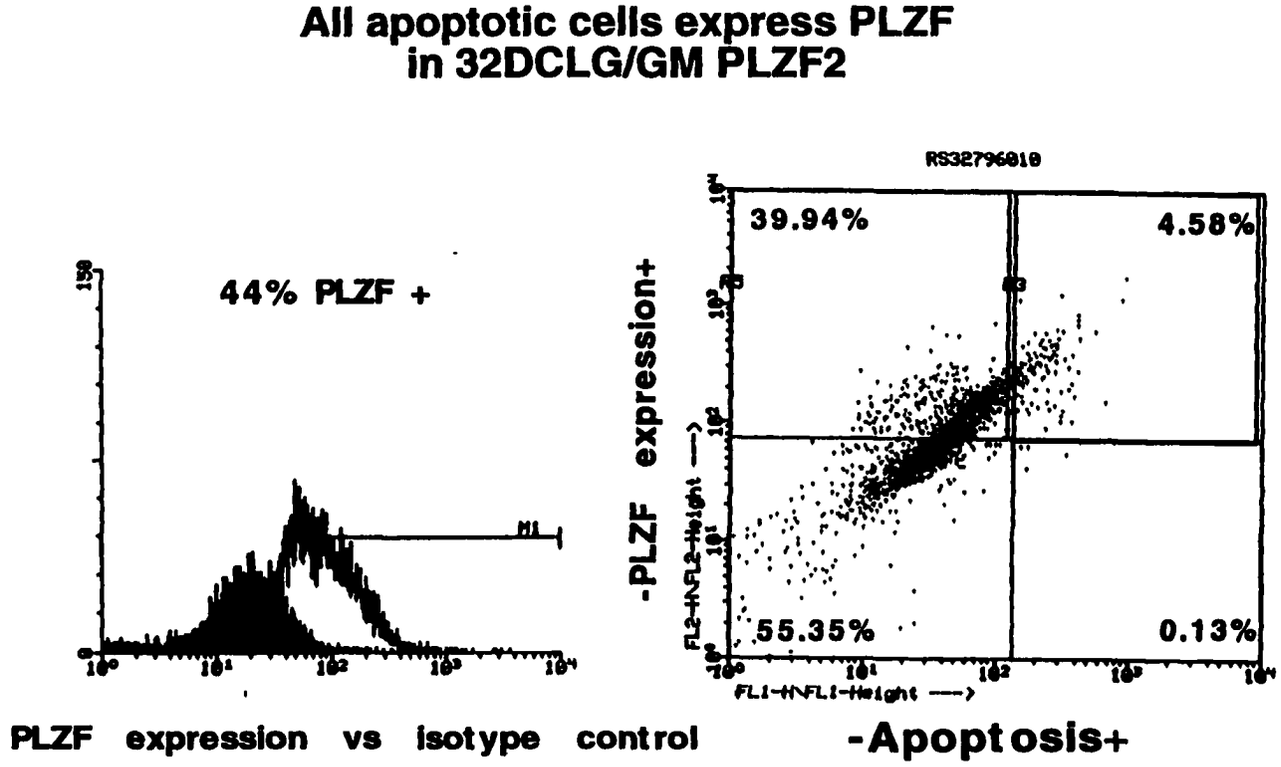


Figure 17. FACS analysis of 32DG/GM PLZF2 pool. Cells were double stained against PLZF and apoptosis. In the left panel determination of PLZF positivity is demonstrated. In the right panel distribution of cells based on PLZF and apoptosis stainings is shown along FL2 and FL1 axis. Percentage is indicated

Figure 17 in the corners of 4 panels.

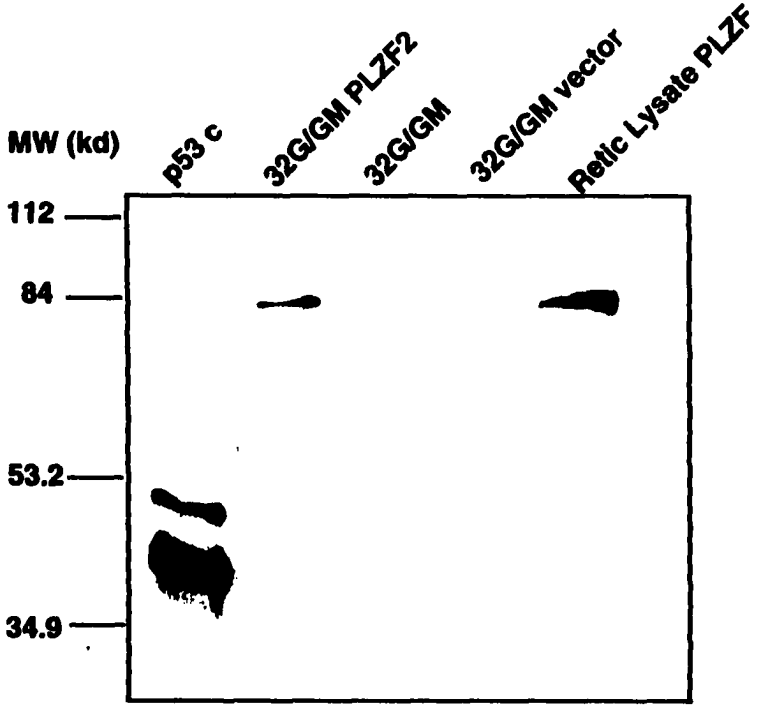


Figure 18. Apoptosis induced with cytokine deprivation does not lead to upregulation of p53. 32DG/GM vector, Wild type and PLZF 2 pools were grown in the absence of IL 3 for 48 hours, collected and whole cell extracts were separated on 10% SDS PAGE, transfered to PVDF immobilion membrane and blotted with affinity mouse ant-PLZF antibody and anti-p53 antibody.

Figure 18

Figure 19. Effect of PLZF on expression of the IL-3 Receptor. (A). Western Blot analysis of the whole cell lysates from Vector and PLZF 2 cells. Monoclonal anti-PLZF antibody and anti-IL-3 Receptor α antibody were used. FACS analysis of IL-3 Receptor expression in infected and uninfected cell pools. Cells maintained in IL-3 were harvested and stained with an anti-IL-3 Receptor antibody, that specifically recognizes the α subunit of mIL-3 receptor α.The bar graph displays the average and range of the percent of IL-3 Receptor positive cells from two independent experiments.

Figure 19

CHAPTER VII

PLZF transforms 32DG/GM cells into earlier progenitor-like cells and blocks their differentiation.

INTRODUCTION

I created 32DG/GM PLZF cell pools to study the effect of PLZF on myeloid differentiation. To do this 32DG/GM cells were cultured in the presence of G-CSF and GM-CSF that normally lead to granulocytic and monocytic differentiation. I assessed morphology and changes in cell surface markers in experimental pools in order to determine the state of differentiation. The cellular morphology is usually assessed using histochemical stains. The stains that are frequently used for blood cells consist of mixture of acidic and basic dyes. In Wright's stained cytospins the leukocyte's nuclei are stained metachromatically with methylene azure and the cytoplasm of lymphocytes and monocytes are stained blue with methylene blue, the neutrophilic granules are stained with eosinate of methylene azuire and usually have lavander colour (Copenhaver et al., 1978). Therefore the morphology is assessed based on the colour and the appearence of the cellular components. In differential counts the myeloid cells are subdivided on the basis of the nuclear differentiation. The commonly used classification for neutrophils, proposed by Schilling, divides neutrophils into segmented and nonsegmented. As a marker of undifferentiated state Sca-1(LY-6A) antigen was used. Sca-1 is a

phosphatydylinositol-anchored protein of 18kDa, which is expressed on multipotent hematopoietic stem cells in mice. Thy-1^{lo}, Lin^{-} (do not express markers characteristic of B cells, granulocytes, myelomonocytic cells and T lymphocytes), Sca^{+} subpopulation of the mouse bone marrow contains all stem cells of the mouse (Rijn et al., 1989). As a late differentiation marker I used myeloid differentiation marker GR-1 (Ly-6G). When bone marrow cells were sorted into Gr-1-negative and positive cells, only Gr-1 negative cells responded to IL-3 and CSF with forming colonies. Gr-1 expression correlated with more mature granulocytic phenotype and diminished ability to proliferate in response to the growth factors. (Fleming et al., 1993).

RESULTS

PLZF overexpression prevents terminal granulocytic and monocytic differentiation. 32DG/GM cells undergo granulocytic and monocytic differentiation in response to G-CSF and GM-CSF respectively. The morphology of various cell pools is demonstrated in Figure 20. When grown in IL-3, cells from all pools, including those expressing PLZF, show immature morphology characteristic of myeloblasts with round nuclei and a large nuclear to cytoplasm ratio. Wild type and vector pools show morphologic evidence of maturation after being exposed to G-CSF and GM-CSF. The nuclei of these cells become lobulated or bean-shaped and smaller in proportion to the cytoplasm, and in GM-CSF characteristic ring-nucleated murine neutrophils appear. In contrast, PLZF overexpressing pools showed few signs of differentiation, with morphology being relatively unchanged (Figure 20 and data not shown). The differential counts of the

cytospin slides indicate that PLZF pools reach only 30-40% differentiation in G-CSF or GM-CSF, while wild type and control pools reach 85-90% of differentiation in the presence of the differentiation factors (Figure 21). The state of differentiation was confirmed by examination of the cell surface markers. The percentage of cells expressing Sca-1 (Figure 22) in the vector pool was 20%, while PLZF pools had up to 75% of cells expressing Sca-1 in IL-3. After the addition of G- or GM-CSF these pools upregulated Sca-1 by another 10%. The intensity of Sca-1 staining was up to two times higher in PLZF pools (Figure 23A). On the other hand the pattern of expression for the maturation marker GR-1 (Figure 22) showed the opposite pattern with 55% of Vector cells staining positively, reflecting relatively differentiated state of G/GM subline. The percentage of positive cells rose to 75 after exposure to G-CSF for two weeks. In PLZF pools basal Gr-1 expression varied from 5 to 25% and did increase by 5-10% after G- or GM-CSF treatment. Gr-1 intensity in PLZF pools was on average two times lower than in Vector and uninfected cell pools (Figure 23B).

PLZF increases survival of 32DG/GM cells after IL-3 withdrawal

Since PLZF is expressed in quiescent and apoptosis resistant $CD34^+$ progenitor cells (Reid et al., 1995) we studied the effect of PLZF overexpression on cell death induced by withdrawal of IL-3. Within days after IL-3 withdrawal wild type and Vector cells underwent apoptosis, characterized by an increased subG1 peak in DNA content and an increased labeling with terminal transferase of 3'-OH ends of DNA (data not shown). Since PLZF cells have a higher incidence of apoptosis when

cultured in the presence of IL-3, we expected to see much faster induction of the death program in PLZF expressing pools after IL-3 withdrawal. To our surprise PLZF expressing cells survived longer in the absence of the growth factor than the control cells (Figure 24A). Both wild type and Vector cells were all dead after one week of growth in the absence of IL-3. In contrast, PLZF expressing pools of 32DG/GM cells had more than 10% of their population alive after 2 weeks of growth without the factor. Live cells, seen on the cytospins, have characteristic 32DG/GM morphology (Figure 24B). Surviving cells were blocked in G1 phase of the cell cycle, as determined by FACS analysis of the DNA content of cells withdrawn from IL-3 for 4 days. (Figure 15).

DISCUSSION

PLZF had significant inhibitory effect on differentiation of 32DG/GM cells in this study. It was suggested that differentiation of myeloid cells usually occurs in two steps and requires G1 arrest (Yen et al., 1987; Yen et al., 1994). Since 60-80% of 32DG/GM PLZF cells were already arrested in G1 before the action of differentiation factors the lack of differentiation in PLZF lines can not be explained by inability to undergo G1 arrest. Although it is possible that PLZF pools are not capable of proliferation that is required for differentiation commitment. It is also possible that PLZF prevents expression of genes determining the lineage commitment and thus maintains cells in undifferentiated state. PLZF may also affect the expression of the cytokine receptors like G-CSF or GM-CSF and render

cells unresponsive to the differentiation factors. Diifferentiation of the vector cell pool was robust with up to 75% of cells having mature morphology after two weeks of differentiation and 50 % of cells expressing Gr-1. PLZF expressing pools never exceeded 35% differentiation based on morphology and had very striking changes in expression of the cell surface markers. PLZF expression was associated with significantly increased expression of Sca-1 and decreased Gr-1 expression, leading us to conclude that PLZF forces cells into a more immature state. This is consistent with the fact that PLZF is expressed in $CD34^+$ cells and is possibly necessary for the maintanence of the dormant progenitors. The inability of PLZF expressing pools to respond to differentiating agents used in our study may be due to the G1 block that PLZF produces and to the inability of PLZF pool to undergo necessary proliferation. At the same time the effect of PLZF on 32DG/GM cells is not limited to the cell cycle, since even when grown in IL-3 these cells have a more immature phenotype. This suggests that PLZF may have control over regulators of differentiation.

PLZF was shown to protect cells from apoptotic death after IL-3 withdrawal. PLZF has a long term effect that persisted for up to two weeks and demonstrated to protect up to 10% of the population from apoptosis. The effect of PLZF is very similar to the effect of bcl-2, which when overexpressed in 32Dcl3 cells retards apoptosis after IL-3 deprivation. It is possible that decreased sensitivity to IL-3 withdrawal is the result of the diminished numbers of IL-3 Receptors on the cell surface and diminished dependence of cells on IL-3 for survival. Anti-apoptotic function of PLZF may be necessary to protect hematopoietic progenitor cells from cell death

after they are arrested in G0 and have to persist for a long time. Strikingly, bcl-2 overexpression also inhibits differentiation of 32Dcl3 cells before and after IL-3 withdrawal (Lin et al., 1996), even though it seems not to interfere with differentiation in some other cell lines. Protection from apoptosis by itself is not sufficient to inhibit differentiation, since overexpression of bcl-2 family member A1 protects 32Dcl3 from cell death, but has no effect on their differentiation. Seemingly opposite is the ability of PLZF to increase the rate of apoptosis in cells (Chapter VI) maintained in IL-3. We think that this effect is the result of PLZF overexpression in significantly differentiated cell line 32DG/GM, where it creates the conflict of undifferentiating the cell line that was commited to differentiiation by the previous exposure to G-CSF.

It is very interesting that DR-nm23, that was cloned because of its expression in the blast crisis of CML, causes block of granulocytic differentiation and increased apoptosis when overexpressed in 32Dcl3 cell line (Venturelli et al., 1995). The striking similarity between DR-nm23 and PLZF is that they are highly expressed in purified $CD34^+$ cells and when overexpressed cause similar phenotype. From the results in Chapters VI and VII that demonstrated that PLZF causes growth suppression, G1 arrest, block in differentiation and protection of cells from apoptosis in the absence of IL-3, it is possible to propose that PLZF is an early hematopoietic factor that is necessary for the maintanance of the progenitor pool in an undifferentiated state.

Figure 20. PLZF overexpression inhibits ability of 32DG/GM cells to differentiate in response to G-CSF or GM-CSF. (A). 32DG/GM cells were grown in the presence of IL-3, G-CSF or GM-CSF for two weeks, then cells were washed and centrifuged onto the slides, air dried and stained with modified Wright-Giemsa. The representative fields are shown in the figure. (B). The morphology of the cells was assessed and each cell was scored as undifferentiated or differentiated based on nuclear/cytoplasmic ratio and nuclear shape. At least 300 cells were counted on each slide and the percentage differentiated cells was plotted. Data from two independent experiments are presented.

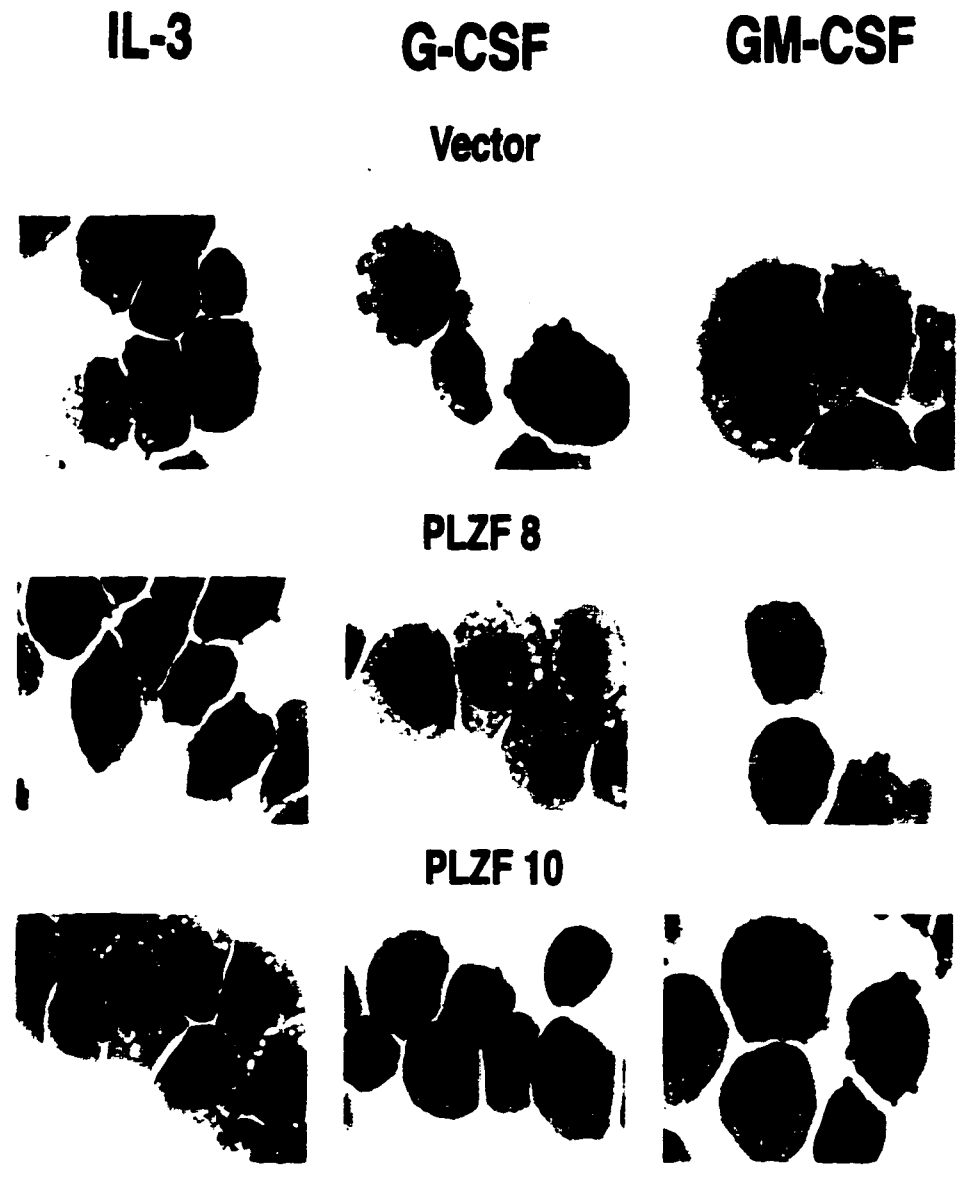


Figure 20

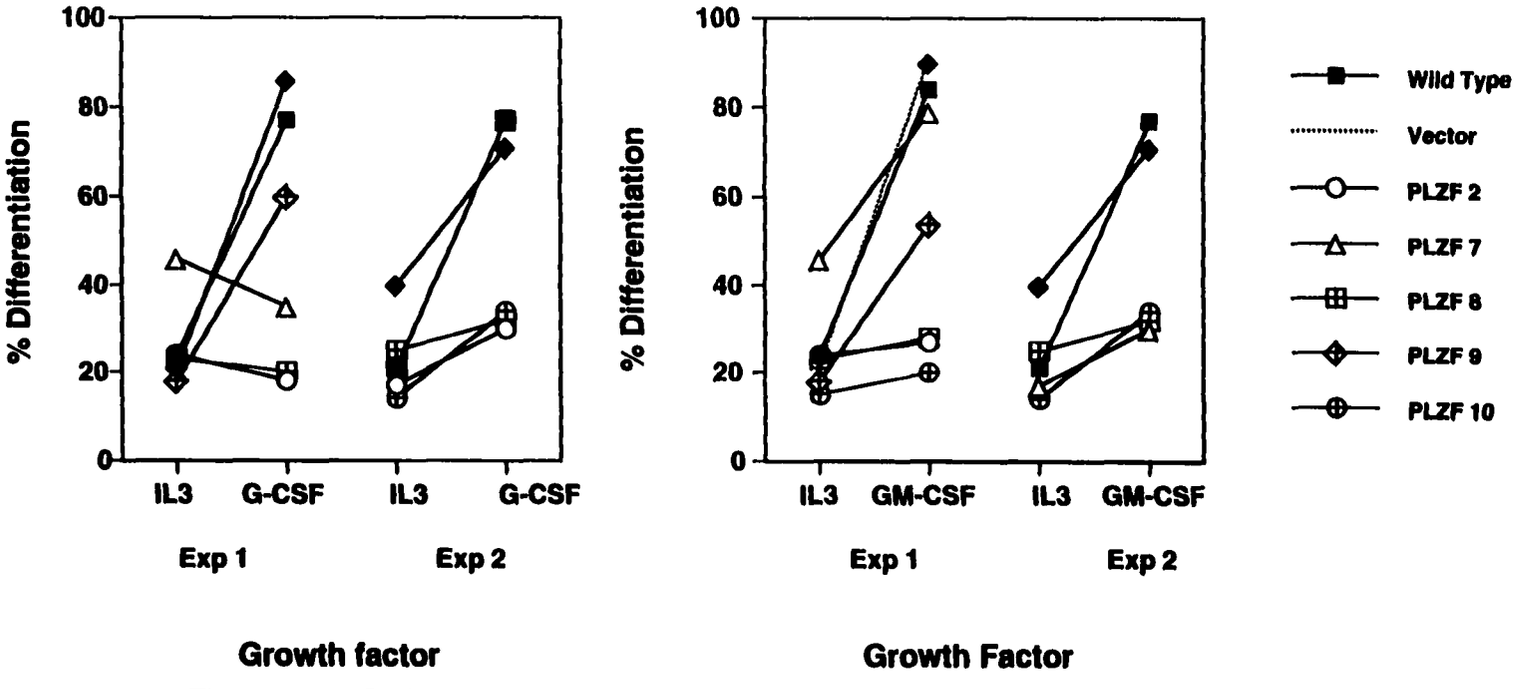


Figure 21. Differentiation block produced by overexpression of PLZF. The morphology of the cells was assessed and each cell was scored as undifferentiated or differentiated based on nuclear/cytoplasmic ratio and nuclear shape. At least 300 cells were counted on each slide and the percentage differentiated cells was plotted. Data from two independent experiments are presented.

Fig22Expression of the cell surface differentiation markers in control and PLZF pools. Cells were maintained in IL-3, G- or GM-CSF for two weeks, harvested and stained with anti-Sca-1 or anti-Gr-1 antibody. Cells were analyzed by FACS. The percentage of cells positive for the expression of cell surface markers indicated and the standard deviations are represented in the figure.

Figure 23. Effect of PLZF expression on the Intensity of Sca-1 and Gr-1 expression. Cells were grown and stained as described in Figure 22. The relative intensity of Sca-1 and Gr-1 staining reflects the density of the antigen expression on the cell surface. It was derived independently for each sample by dividing the median intensity of staining with specific antibody by the median intensity of staining with an isotype control.

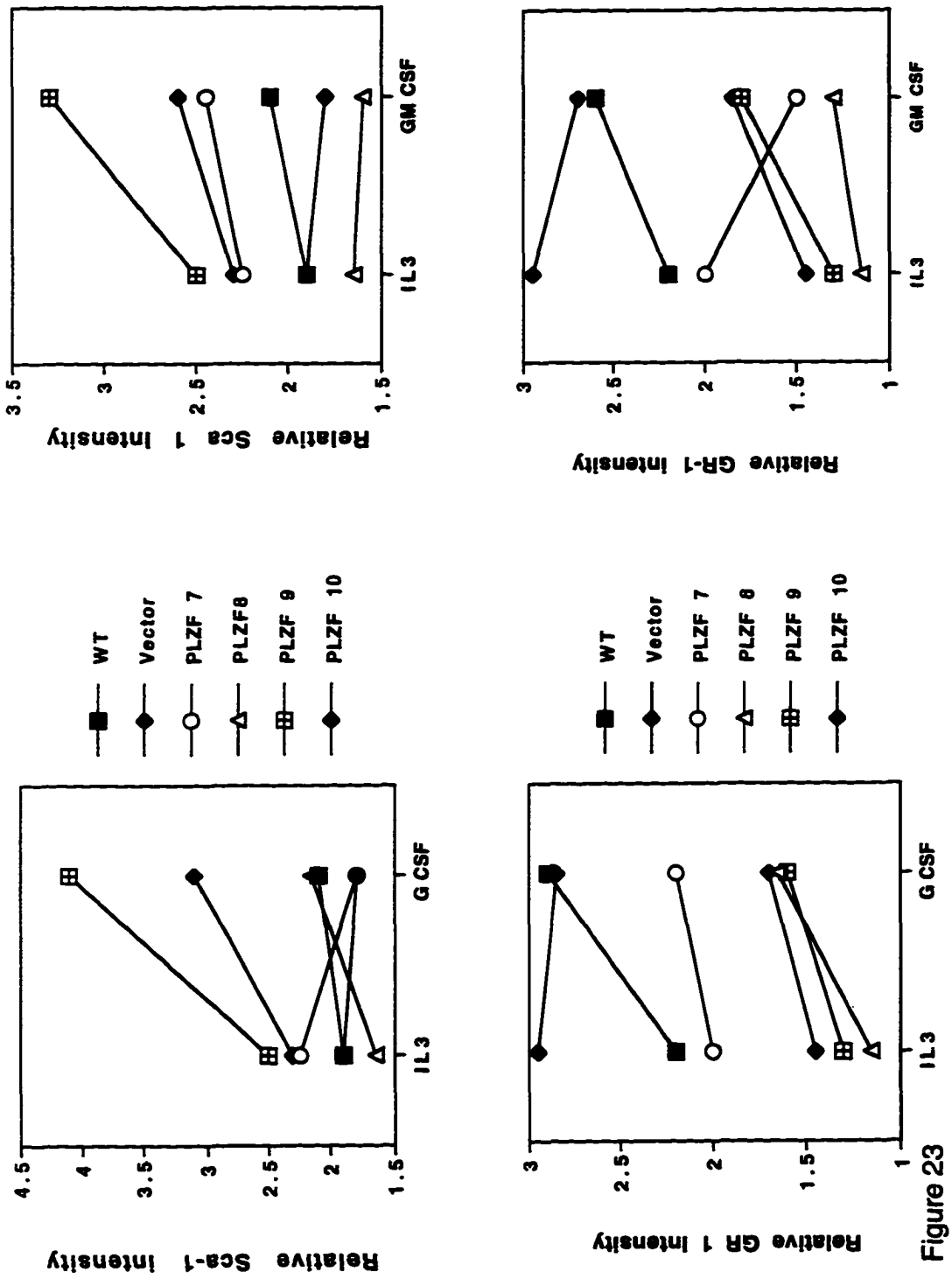


Figure 23

Figure 24. PLZF prolongs survival of 32DG/GM cells after IL-3 withdrawal. (A). Cells were washed free from IL-3, plated in culture medium without IL-3 supplementation, and live cells were counted by Trypan Blue exclusion. (B). Morphology of cells maintained for 2 weeks without IL-3. Cells were centrifuged onto slides, air dried and stained with a modified Wright Giemsa stain.

Figure 24

CHAPTER VIII

Stable expression of PLZF induces production of the growth inhibitory substance in 32DG/GM cells.

INTRODUCTION

The suppressive effect of PLZF overexpression on the growth of 32DG/GM cells was dramatic even though according to our FACS data (Figure 17) PLZF is expressed in less than 50% of cells in a pool at any given time. Therefore we hypothesized that PLZF pools of 32DG/GM cells could secrete an inhibitory factor into the growth medium. To detect the growth inhibitory activity secreted into the growth medium the medium was used to culture the Vector pool and measure the effect on cell proliferation.

RESULTS

Conditioned medium from PLZF expressing cells suppresses cell growth. 32DG/GM cells infected with the insertless reporter were cultured in 50% conditioned medium. Cells cultured in medium conditioned by PLZF expressing cells were growth inhibited, reaching a 50% lower peak density and exhibiting an increase in estimated doubling time (Figure 25 A). Contamination with PLZF expressing cells was

eliminated by filtering the CM. We also demonstrated that infected 32DG/GM cells did not package retroviruses since the CM failed to confer puromycin resistance to NIH 3T3 cells (data not shown).

When CM was taken from exponentially growing 32DG/GM PLZF cells the effect of CM on the growth of control cells was more pronounced (Figure 25B). The growth suppressive effect was strongest when cells were cultivated with 50% CM and diminished with 20 and 10% CM. CM from the Vector pool did not suppress growth.

DISCUSSION

In normal hematopoiesis most of the stem cells are in non-proliferative state. It could be a passive process resulting from the absence of the positive signals or the active process engaging the inhibitory regulators. There are several cytokines that are known to inhibit proliferation of the target cells in culture, among them interferons (IFNα, β, γ), transforming growth factor β, macrophage inflammatory protein -1α and several others. Most of these cytokines cause cell cycle arrest in G0/G1 phase by effecting expression of c-myc, cyclin A or pRB phosphorylation (Kimchi, 1992). Since overexpression of PLZF leads to G1 arrest in 32DG/GM cells and at the same time results in detectable autocrine growth inhibitory activity it is

very likely that PLZF directly or indirectly activates one of the genes coding for an inhibitory cytokine. One likely candidate is Transforming growth factor-β (TGF-β), which is likely to be a physiologic regulator of early hematopoietic cells. TGF-β is known to be secreted by hematopoietic progenitors and is likely to be activated in the stable 32DG/GM PLZF pools since PLZF renders these cells phenotypically immature. TGF-β was shown to be highly pleiotropic in myelopoiesis, stimulating day 7 CFU-GM, but inhibiting day 14 CFU-GM, as well as early murine bipotent and multipotent CFU (Ottman and Pelus, 1988). Addition of TGF-β to long-term bone marrow cultures inhibited proliferation in response to change of media or addition of IL-1 (Cashman et al., 1990). It is intriguing that one of the mechanisms of TGFβ action is postulated to be down-regulation of IL-1 receptor expression which leads to the inability to respond to positive cytokines (Dubois et al., 1990). Since IL-3 receptor is not expressed on early progenitors its expression may be inhibited by TGF-β, among other factors. Thus down-regulation of IL-3 receptor in 32DG/GM pools may be accomplished by several mechanisms: through direct or indirect action by PLZF on the IL-3 Receptor promoter or by PLZF-induced expression of an inhibitory cytokine (TGF-β or even a novel kind) that dictates the stage-appropriate cytokine-receptor' expression.

Our lab is developing preliminary data suggesting that PLZF may bind the promoter of IL-6 to regulate its activity (Dr. Helen Ball, unpublished data). IL-6 inhibits growth of some cells through multiple pathways. In M1 myeloblastic cell line IL-6 represses transcription of c-myc through an element between positions -65 and -58 by suppressing binding of E2F (Melamed et al., 1993). Cells constitutively expressing c-myc did not undergo G0/G1 arrest in response to IL-6 treatment, suggesting that c-myc is essential in IL-6 signalling (Melamed *et al.*, 1993). The secretion of an inhibitory cytokine by PLZF expressing cells fits very well with the phenotype of quiescence and dedifferentiation characterizing PLZF pools. It also opens up a new direction in research of biological functions of PLZF and raises many questions about the nature of an inhibitory factor, its specificity and the mechanism of action.

Figure 25. PLZF expressing pools secrete a growth inhibitory factor. 32DG/GM cells infected with empty retroviral vector were plated at $1x10^5$ cells per ml in the presence of CM. Upper panel represents the effect produced by 50% conditioned medium. The lower panel displays the effect of different dilutions of the medium from exponentially growing experimental cultures on 32DG/GM Vector cells. Live cells were counted by trypan Blue exclusion.

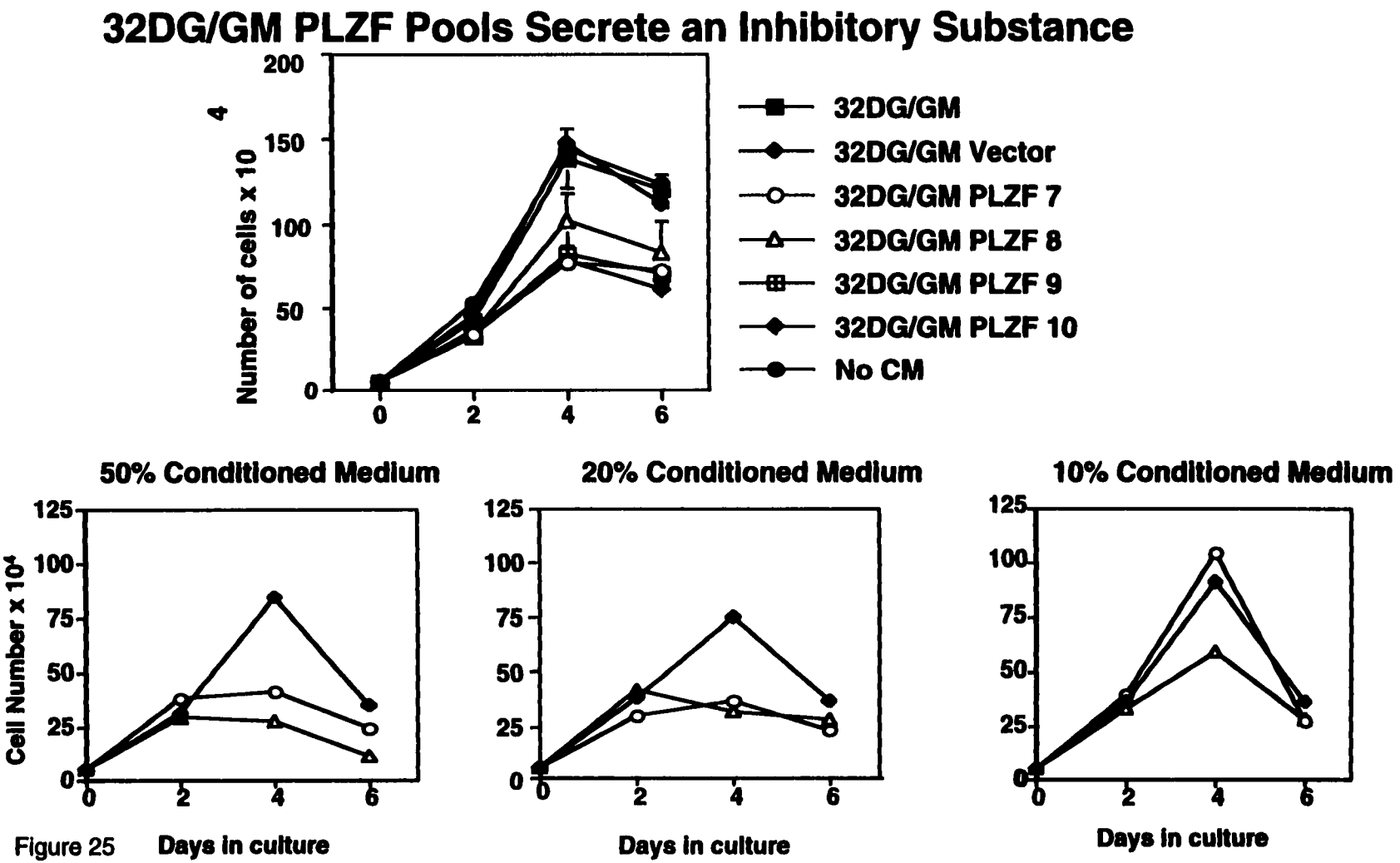


Figure 25

Chapter IX

Conclusions

The current understanding of hematopoiesis is that differentiation of hematopoietic lineages involves a network of transcription factors and that disruption of these factors leads to defects in hematopoiesis. Chromosomal translocations involving these factors are often associated with hematologic malignancies. PLZF is a novel transcription factor that has been cloned as a result of its involvement in APL associated with translocation t(11;17) (Chen et al., 1993). Later it was demonstrated that PLZF is differentially expressed in hematopoietic lineages and is highly expressed in early $CD34^+$ hematopoietic precursors (Cook et al., 1995; Reid et al., 1995). On the basis of this information I decided to pursue the characterization of PLZF protein and of its biological functions in hematopoietic development.

In Chapter III, I characterized the monoclonal and polyclonal antibodies raised against PLZF. I demonstrated that Hybridoma #2A had the strongest interaction with PLZF and recognized N-terminal domain between amino acid 100-200.

These antibodies proved to be a very useful tool to study PLZF. Using these antibodies I demonstrated that PLZF is a 90 kDa protein that localizes in nuclear bodies in HL-60 and MDS cells and gives homogenous nuclear staining when overexpressed in CV-1 cells. I also showed that in transient transfections in CV-1 cells PLZF-RARα and RARα-PLZF are mostly nuclear proteins that do not change their localization after treatment with ATRA. PLZF is phosphorylated on Ser and Thr that are present throughout the length of the protein. Nuclear localization and phosphorylation are important characteristics of the transcription factor, that can be used to study the regulation of its biological activity. Since now there are several candidate PLZF DNA binding sites (Li *et al.*, 1997; Yeyati *et al.*, 1997), it is possible to study the effect of phosphorylation on the DNA binding activity of PLZF and, also, to identify whether cyclin A cdk2 complex or MAPKinase, which were suggested as candidate kinases, can phosphorylate PLZF. Others in the laboratory used these antibodies to identify PLZF presence in DNA-binding complexes in the EMSA (Licht *et al.*., 1996). Anti-PLZF antibodies were also used to demonstrate expression of PLZF in $CD34^+$ cells and to describe the temporal and spatial pattern of PLZF expression, allowing to predict the significance of PLZF for control of the cell cycle and for the development of hematopoietic and nervous systems.

In Chapters V, VI and VII, I described the effect that overexpression of PLZF had on the myeloid cell line 32DG/GM. PLZF caused GI arrest, inhibition of cell growth and increased cell death when overexpressed. These effects suggested that PLZF may control the cell cycle. The cell cycle effect of PLZF in 32DG/GM cells opened a new direction in PLZF research. Dr. P. Yeyati focused on cyclin A, which is necessary for G1 to S transition, as a potential target of PLZF. The results suggest that cyclin A expression is directly down-regulated by PLZF, while RARα-PLZF may upregulate cyclin A expression and lead to increased proliferative potential in the cells and may contribute to leukemogenesis in t(11;17) APL. It would be very useful to develop a transgenic model for t(11;17). The mice transgenic for PLZF-RARα and RARα-PLZF expression may develop APL, based on the effect PLZF has on the cell cycle. The leukemic phenotype may require the expression of both reciprocal fusion proteins, since disregulation of RARα responsive genes as well as PLZF responsive genes probably contribute to leukemogenesis in t(11;17) patients.

PLZF also inhibited differentiation of 32DG/GM cells in response to G-CSF and GM-CSF. Its expression correlated with upregulation of a primitive hematologic Sca-1 marker and down-regulation of the granulocytic Gr-1 marker; down-regulation of IL-3 receptors, which provides important

proliferative potential to the committed hematopoietic cells; prolonged survival of PLZF-expressing pools in the absence of IL-3. I also found that PLZF expression was associated with secretion of the autocrine growth-inhibitory factor. These findings led us to believe that PLZF is a transcription factor that is important for maintaining the hamatopoietic progenitors in quiescent state, since PLZF confers more primitive phenotype to 32DG/GM cells, blocks them in G1/G0 phase of the cell cycle and makes them unresponsive to proliferative cytokines like IL-3 and to differentiation cytokines like G-CSF and GM-CSF. This hypothesis is supported by expression of PLZF in $CD34^+$ hematopoietic precursors. In addition the preliminary results from the analysis of mice homozygous null for PLZF expression show that these mice develop leukopenia and exhaust their stem cell population as they grow older. These mice are also sterile with disrupted spermatogenesis. It is possible to hypothesize that PLZF maintains a pool of undifferentiated spermatogonia, similarly to its action in hematopoietic progenitors (Dr. P .P .Pandolfi, unpublished data). It is an exciting finding in the light of the fact that it is still unclear what maintains the pool of stem cells. It leads to several questions. Is PLZF necessary for the maintenance of the stem cell population? Will overexpression of PLZF in a pool of more committed and numerous hematopoietic progenitors in bone marrow replenish the stem cell? And if yes, can this strategy be used as a gene therapy in bone marrow transplants? Is regulation of cyclin A by PLZF a foundation for the broader effect that PLZF knock-out has on

hematopoiesis and germ cell development? All these questions are intriguing and relevant in the context of the findings described in this thesis work.

Chapter X

Model of the role PLZF plays in hematopoiesis and APL.

PLZF is a key regulatory factor expressed in a small pool of hematopoietic stem cells. As was presented in the above thesis study, PLZF blocks cells in the G1 phase of the cell cycle and maintains their undifferentiated phenotype. These results allow one to propose a model whereby PLZF is responsible for the maintanance of the small population of self-renewing hematopoietic precursors in an undifferentiated and unproliferative state throughout adult life without premature expansion and commitment to any hematopoietic lineage (Figure 26). This model for PLZF function is strongly supported by the phenotype of the PLZF knock out mice that develop leukopenia, which possibly signifies the exhaustion of the hematopoietic stem cell pool (Dr.Pandolfi, unpublished data) .

Consistent with this model I have demonstrated that overexpression of PLZF blocks cells in G0/G1 phase of the cell cycle, probably through the same molecular mechanisms it utilizes to produce quiescence when expressed in the population of the stem cells. The mechanism by which PLZF affects the cell cycle was elucidated by Dr P. Yeyati. Dr.P.Yeyati demonstrated that PLZF may directly bind to the cyclin A promoter and

repress it in *in vitro* systems. She also established that overexpression of PLZF is correlated *in vivo* with down-regulation of cyclin A expression. It is tempting to postulate that regulation of cyclin A may involve phosphorylation of PLZF itself. When unphosphorylated, PLZF may interact with cdk2 and in this complex may bind and repress cyclin A promoter but not its own promoter. Under the influence of other factors, cells undergo commitment to proliferate, PLZF may be phosphorylated followed by the release of cdk2 and upregulation of cyclin A levels. This chain of events will lead to G1-S transition and exit of the cell from G0.

From my results it can also be postulated that PLZF may contribute to cell cycle arrest by maintaining Rb in an underphosphorylated state and by activating secretion of the growth inhibitory factor X, which acts in the autocrine manner. The described repression of hIL3 receptor by PLZF may be responsible for the low level of IL-3 receptors on the cell surface of the stem cells and for their unresponsiveness to proliferative stimuli by IL-3.

In this model, PLZF prevents differentiation of the stem cells. It is logical to suggest that the lineage specific genes like globin, cd14 and myeloperoxidase may be directly repressed by PLZF, while cd34 and sca-1 may be directly activated by PLZF. The immature phenotype may be maintained by repressing expression of the cytokine receptors: for example G-CSGF R and GM-CSF R. It is possible that when cells commit to exit the

dormant stage and undergo expansion expression of PLZF is downregulated, but not eliminated. This leads to formation of numerous BFUs and CFUs, that overcame the growth suppressive effect of PLZF. It is possible that control of the cell cycle requires high concentration of PLZF. For example, cyclin A positively regulates its own promoter, possibly quickly escaping PLZF control after PLZF is downregulated. At the same time at the stage of BFUs lower concentrations of PLZF are sufficient to prevent terminal differentiation of these cells guaranteeing their expansion. We propose that if at this stage a cell is exposed to the high concentrations of differentiation factors, it already can perceive the signal due to the rising density of the cell surface receptors, but is unable to commit to differentiation due to PLZF expression, and thus the clash of signals leads to the programmed cell death. At the normal physiological levels of cytokines the hematopoietic precursors continue to expand and finally exit the bone marrow compartment into the peripheral blood. According to the model, peripheral blood cells that are not terminally differentiated contain no PLZF, have high level of IL-3 receptor and start expressing lineage specific markers. Under further influence of differentiation cytokines, cells commit to a specific lineage and undergo differentiation. Lineage specific transcription factors like PU.1 or GATA-1 may repress PLZF directly. It is possible that in terminally differentiating macrophages expression of PLZF reemerges: it may again secure the

G1 arrest necessary for terminal differentiation, or activate macrophage specific genes that became accessible on the chromosome for direct transcriptional control.

The rearrangement of PLZF in t(11;17) may lead to the loss of cell cycle control and to differentiation defect. APL cells with t(11;17) express RAR-PLZF, PLZF-RAR and reduced levels of RAR and PLZF proteins. PLZF-RAR binds RAR-responsive genes and as a result interferes with the function of RAR and may be contributing to the block of differentiation by disregulating the hox genes. On the other hand RAR-PLZF can still bind PLZF -responsive genes, including the cyclin A gene. However, instead of inhibiting, it activates them due to the A1 domain of RAR. Increased levels of cyclin A drive cells from G1 to S and shorten the cell cycle. Treatment with RA raises concentrations of RAR-PLZF, which is under the control of the RA-responsive promoter of RAR, and leads to the loss in cell cycle control and expansion of the malignant clone. Uncontrolled proliferation prevents terminal differentiation that requires growth arrest. RAR-PLZF titrates out not only PLZF, but also RXR, which is necessary for wild type RAR function. Thus RAR-PLZF has a dominant-negative effect on both RAR and PLZF wild type proteins and is probably a key offender in t(11;17) APL.

The role of PLZF in Hematopoiesis

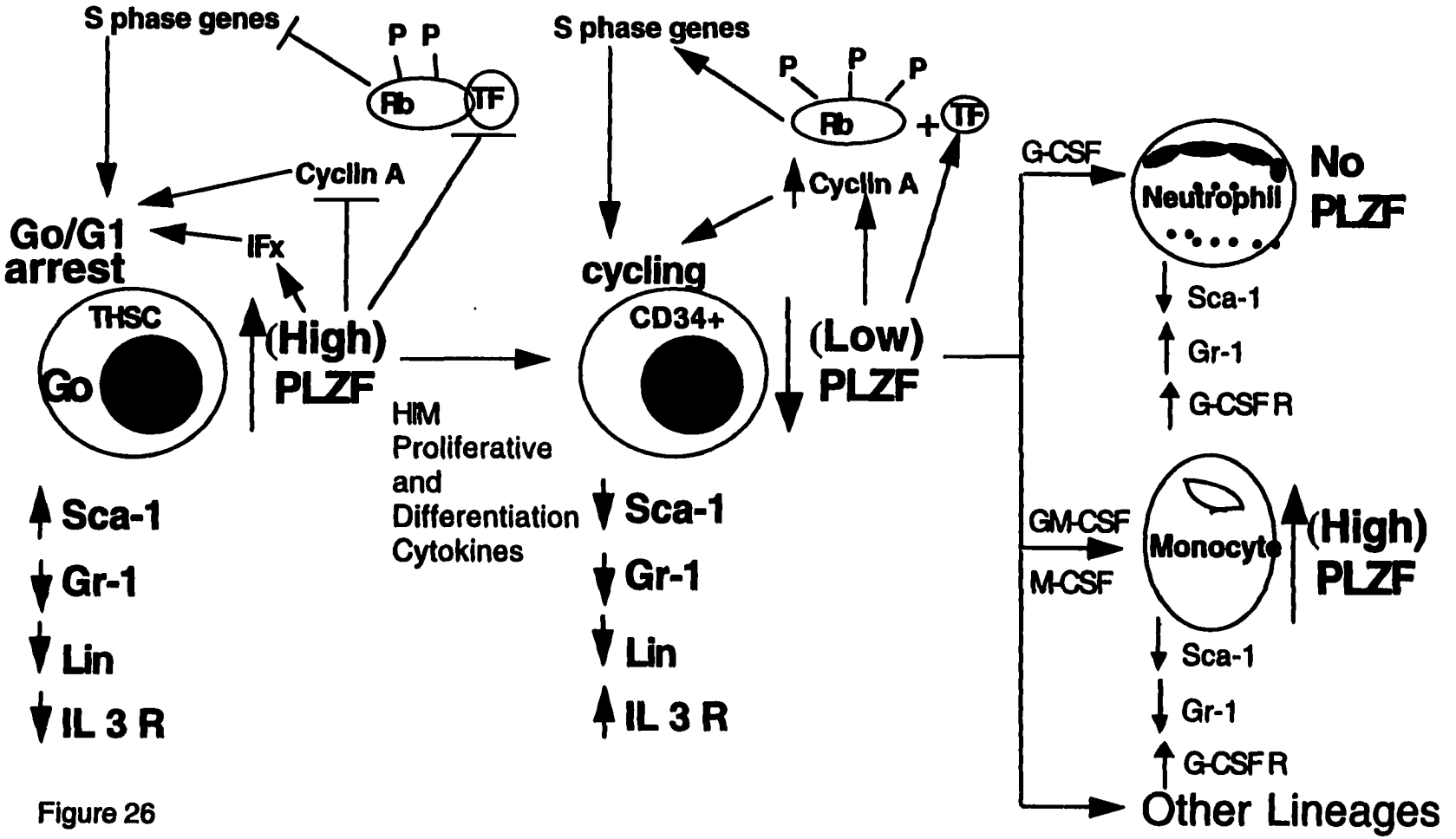


Figure 26

Chapter XI

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