Oncogene (2002) 21, 6669–6679

www.nature.com/onc

PLZF induces megakaryocytic development, activates Tpo receptor expression and interacts with GATA1 protein

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We investigated the expression of the PLZF gene in purified human hematopoietic progenitors induced to unilineage erythroid, granulocytic or megakaryocytic differentiation and maturation in serum-free culture. PLZF is expressed in quiescent progenitors: the expression level progressively rises through megakaryocytic development, whereas it gradually declines in erythroid and granulopoietic culture. To investigate the role of PLZF in megakaryopoiesis, we transduced the PLZF gene into the erythro-megakaryocytic TF1 cell line. PLZF overexpression upmodulates the megakaryocytic specific markers (CD42a, CD42b, CD61, PF4) and induces the thrombopoietin receptor (TpoR). The proximal promoter of the TpoR gene is activated in PLZF-expressing TF1 cells: in this promoter region, a PLZF DNA-binding site was identified by deletion constructs studies. Interestingly, PLZF and GATA1 proteins coimmunoprecipitate in PLZF-expressing TF1 cells: enforced expression of both PLZF and GATA1 in TF1 cells results in increased upregulation of megakaryocytic markers, as compared to exogenous PLZF or GATA1 alone, suggesting a functional role for the PLZF/GATA1 complex. Our data indicate that PLZF plays a significant stimulatory role in megakaryocytic development, seemingly mediated in part by induction of TpoR expression at transcriptional level. This stimulatory effect is potentiated by physical interaction of PLZF and GATA1, which are possibly assembled in a multiprotein transcriptional complex.
Oncogene (2002) **21,** 6669–6679. doi:10.1038/sj.onc.1205884

Keywords: megakaryopoiesis; PLZF; GATA1; Tpo receptor

Introduction

The promyelocytic leukemia zinc finger PLZF gene was identified by its rearrangement with the retinoic acid receptor α (RARα) in the chromosomal translocation t(11;17)(q23;21) involved in acute promyelocytic leukemia (APL) (Chen *et al*., 1991, 1993a, b; Melnick and Licht, 1999). PLZF-RARα fusion gene has been shown to induce leukemia in transgenic mice (He *et al*., 1998).

PLZF gene encodes a zinc finger transcription factor which is localized in nuclear speckles of $CD34^+$ human progenitor cells (Reid *et al*., 1995), but becomes delocalized in leukemic cells of APL patients (Chen *et al*., 1993a). PLZF is a DNA-binding protein of about 80 to 90 kDa, that can be phosphorylated on serine and threonine residues (Chen *et al*., 1993b; Melnick and Licht, 1999; Reid *et al*., 1995). PLZF belongs to a protein family characterized by the presence of a BTB/POZ domain (Li *et al*., 1997; Bardwell and Treisman, 1994; Ahmad *et al*., 1998) involved in heterodimerization (Dong *et al*., 1996; Koken *et al*., 1997; Davies *et al*., 1999; Hoatlin *et al*., 1999) and colocalization with other oncogenes (Dhordain *et al*., 2000).

A crucial role for PLZF has been suggested in normal development (Cook *et al*., 1995; Ivins and Zelent, 1998) as well as during leukemogenesis (He *et al*., 1998; Hawe *et al*., 1996). Gene targeting studies have recently shown a role of PLZF in the control of murine embryogenesis and in the regulation of HOX gene expression (Barna *et al*., 2000).

In hematopoiesis, the PLZF gene is expressed in myeloid but not lymphoid cell lines (Chen *et al*., 1993a; Melnick and Licht, 1999). In murine embryos, PLZF is expressed in the aorta-gonad-mesonephros region (Melnick and Licht, 1999; Cook *et al*., 1995), where the hematopoietic stem cells were found to emerge from (Godin *et al*., 1993; Medvinsky *et al*., 1993). The molecular pathways through which PLZF exerts its function are still under investigation. PLZF protein has been regarded as a growth regulator (Shaknovich *et al*., 1998), able to inhibit cellular growth by altering the expression of molecules involved in the control of the cell cycle (Yeyati *et al*., 1999). One PLZF target gene reported so far is cyclin A2, whose expression is inhibited by PLZF (Ball *et al*., 1999).

It has also been demonstrated that PLZF exerts a transcriptional repression activity (Melnick and Licht, 1999; David *et al*., 1998) by binding to specific promoter sequences (Li *et al*., 1997), followed by the recruitment of histone deacetylase in the Ncor SMRT-mSin3-HDAC corepressor complex (David *et al*., 1998; Hong

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Received 22 March 2002; revised 16 July 2002; accepted 18 July 2002

et al., 1997; Grignani *et al*., 1998a; Guidez *et al*., 1998; Wong and Privalsky, 1998). Recently, it has been shown that PLZF interacts with the vitamin D3 receptor and may be involved in regulating the 1,25(OH)D3-induced monocytic differentiation in hematopoietic cells (Ward *et al*., 2001). Furthermore, many potential transcription factor binding sites were identified in the putative promoter region of PLZF (Zhang *et al*., 1999b). This suggests that PLZF gene expression may be regulated by a complex network of regulators.

The transcription factor GATA1 has been described to play a major role in hematopoiesis and is implicated in the maturation and differentiation of erythroid and megakaryocytic cells (Orkin, 1992; Pevny *et al*., 1991; Shivdasani *et al*., 1997; Takahashi *et al*., 1998; Vyas *et al*., 1999). GATA1 is also expressed in early progenitors cells (Labbaye *et al*., 1995). GATA1 may exert its biological functions by interacting with other transcription factors to regulate the proliferation/differentiation of hematopoietic cells (Merika and Orkin, 1995; Gregory *et al*., 1996; Wadman *et al*., 1997; Tsang *et al*., 1997; Hung *et al*., 1999; Zhang *et al*., 1999a).

We have investigated the pattern of expression and the possible functional role of PLZF in hematopoietic differentiation. PLZF is preferentially expressed during normal megakaryocyte (MK) development and is upmodulated during MK maturation. Overexpression of the exogenous PLZF gene in TF1 erythro-megakaryocytic cell line induces MK differentiation: in these cells, PLZF interacts with the transcription factor GATA1 and stimulates the thrombopoietin receptor (TpoR) gene transcription, by activating a promoter region which contains a potential DNA binding site for the PLZF protein.

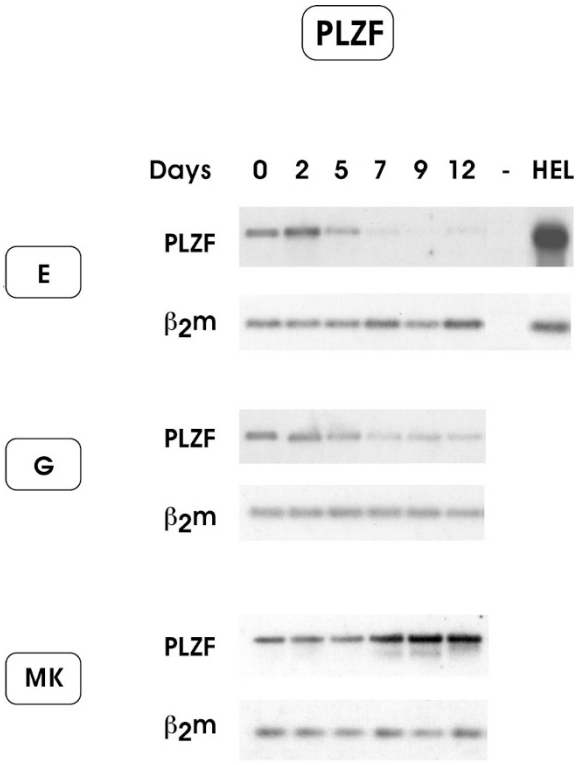

Figure 1 RT–PCR analysis of PLZF mRNA expression in purified HPCs induced to unilineage E, G and MK differentiation in liquid culture and analysed at different culture times. β_2-microglobulin (β_2m) was the internal control. The HEL cell line is used as a positive control. A negative control (−) of the RT–PCR reaction is also shown. Representative results from three independent experiments are shown

Results

Expression of PLZF mRNA in unilineage hematopoietic progenitor cultures

Human hematopoietic progenitor cells (HPCs) were purified from adult peripheral blood according to previously reported procedures (Gabbianelli *et al*., 1990; Labbaye *et al*., 1999). Under appropriate serum-free culture conditions, HPCs undergo unilineage differentiation and maturation along the erythroid (E), granulocytic (G) or MK pathway: in these culture systems, >95% of the cells pertain to a single lineage (Labbaye *et al*., 1995, 1999; Guerriero *et al*., 1995; Testa *et al*., 1996).

To evaluate the expression of PLZF mRNA in the hematopoietic lineages, RNA samples were generated at sequential days from purified HPCs differentiating and maturing through the E, G or MK pathway. The limited cellular availability prompted us to use the RT–PCR method to analyse the expression of PLZF in these cells. Three independent RT–PCR experiments were performed (representative results are shown in Figure 1). A series of controls, including dose-response curves for the assayed templates (not shown) and RT–PCR of the β2 microglobulin gene for the normalization of samples, ensured a semiquantitative evaluation of mRNA levels (Figure 1).

PLZF mRNA is expressed in the quiescent HPCs, and is down-regulated in both E (day 7 of cultures) and G cultures (days 5–7 of cultures), to be expressed at very low levels through terminal E and G maturation (Figure 1). In the MK lineage, PLZF mRNA expression is up-regulated during differentiation from HPCs to the maturation stages from megakaryoblasts to mature polyploid platelet-forming MKs (Figure 1).

Level of PLZF expression, quantificated by densitometric scanning of autoradiograms, shows a threefold decrease during G differentiation and a twofold increase in mature MK cells as compared to undifferentiated HPCs (data not shown), indicating that PLZF expression is regulated during HPCs differentiation according to a lineage-dependent pattern: it is down-modulated during E and G differentiation, while it is up-modulated during MK maturation.

Exogenous PLZF induces MK differentiation in the TF1 cell line

To evaluate the effects of PLZF on hematopoiesis we overexpressed PLZF in the TF1 cell line (TF1-PLZF)

by retroviral transduction. Endogenous PLZF mRNA and protein are barely detectable in wild type TF1 cells (TF1-wt), or in TF1 cells transduced with the empty LXSN vector (TF1-LXSN) (Figure 2a,b). However, in two selected clones (cl 2, cl 13) of TF1-PLZF cells, exogenous PLZF mRNA and protein are both strongly overexpressed, as demonstrated by Northern blot and Western blotting analysis respectively, and as compared to the level of endogenous PLZF protein expression observed in HEL cells (Figure 2a,b).

TF1-PLZF cells grown for several days in the presence of IL-3 exibit a MK-like morphology, and a significant proportion of them are polyploid (data not shown). We could not detect any significant difference as regard the cell growth of TF1-PLZF cells as compared to TF1-wt or TF1-LXSN cells. We analysed the expression of membrane differentiation antigens CD34, CD41, CD42 and CD61 in both TF1-PLZF clones (Figure 3a). As compared to the control of TF1-LXSN cells, TF1-PLZF cells (cl 2, cl 13) presented a slight increase of MK membrane markers (CD41a, CD61), associated with a moderate decrease of CD34 (Figure 3a).

We also evaluated the capacity of PLZF to increase the MK differentiation stimulated by DMSO (Tabilio *et al*., 1984). TF1 cells were grown for 4 days in the presence of DMSO and then analysed for expression of MK markers (Figure 3a). DMSO treatment clearly induced the expression of membrane MK antigens markers CD41a, 41b, 42a, 42b and CD61 in TF1-PLZF cells at levels significantly higher than those observed in the control TF1-LXSN cells (Figure 3a).

It is noteworthy that, while only a part of TF1-LXSN cells displayed MK membranes markers after DMSO treatment, the large majority of TF1-PLZF cells exhibited on their surface MK markers (Figure 3a). This was particularly striking for MK markers which are induced only at late times during MK maturation, such as CD42b, whose expression was low in control TF1-LXSN cells ($<15\%$ positive cells) and high in TF1-PLZF clones ($>95\%$ positive cells) (Figure 3a).

By RT–PCR analysis we found that PLZF expression is up modulated during DMSO- induced TF1 cell differentiation (Figure 3b). This finding was observed in TF1-wt, TF1-LXSN as well as in TF1-PLZF cells. This result is in line with the increase in PLZF expression during MK maturation of normal HPCs. The morphology of these cells showed that about 15–20% of TF1-PLZF cells, but not TF1-LXSN cells, displayed a typical MK morphology, with a polylobulated nucleus and a very large cytoplasm (Figure 3c).

In parallel, we carried out RT–PCR mRNA analysis of MK genes, TpoR (Vignon *et al*., 1992), PF4 (Ravid *et al*., 1991) and EpoR (Maouche *et al*., 1991) in the TF1-PLZF cells (Figure 4). We found that PF4 and TpoR mRNAs expression was markedly up regulated in TF1-PLZF cells, while EpoR mRNA expression was not modified as compared to the mRNA levels observed in the parental TF1 cell line or the TF1-LXSN cells (Figure 4).

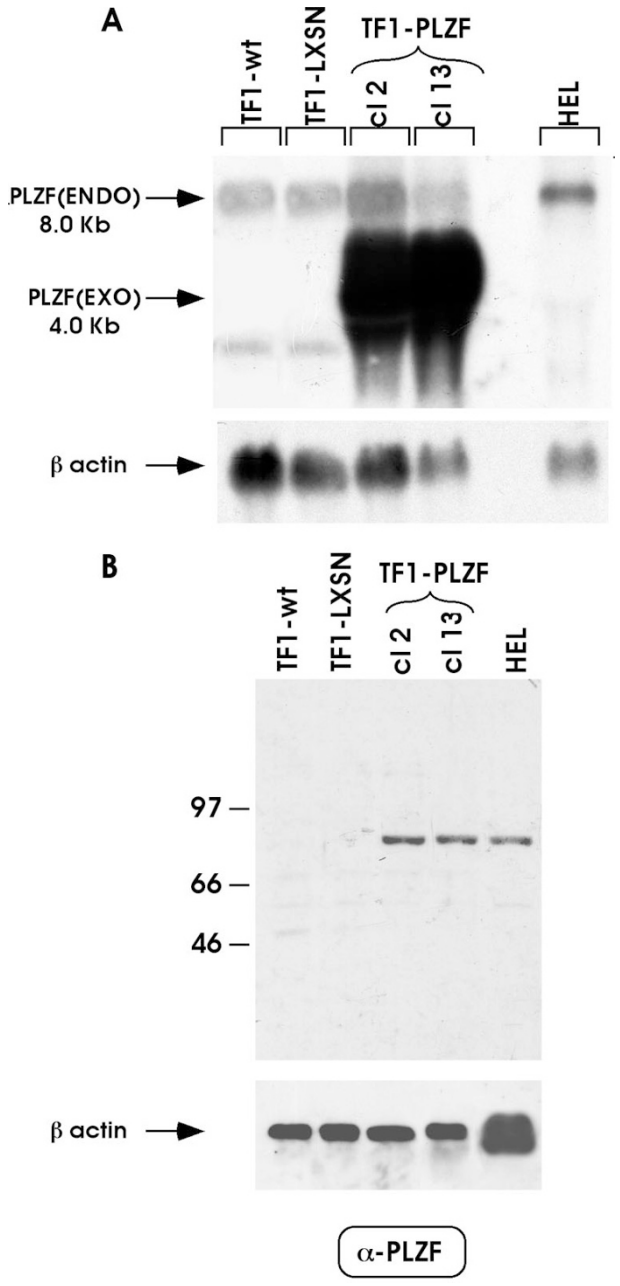

Figure 2 (**a**) Northern Blot analysis of PLZF mRNA expression in TF1-wt; TF1-LXSN, TF1 cells transfected with the empty vector LXSN; TF1-PLZF cellular clones cl 2, cl 13; HEL cells as a positive control. *β*actin (bottom panel) hybridization is shown to assess the quantity of RNA loaded for each sample. Endogenous (ENDO) and exogenous (EXO) PLZF mRNAs are indicated. (**b**) Western blot analysis of PLZF protein expression in nuclear extracts prepared from the cell samples described above. A representative autoradiogram is shown. Molecular weights standard are indicated. The polyclonal antibody *α*-PLZF was used to detect the PLZF protein (MW, around 80 KDa). *β*actin hybridization is shown to assess the quantity of protein loaded for each sample

Taken together, our data show that the enforced expression of PLZF in TF1 cells improves the maturation of these cells to the MK lineage.

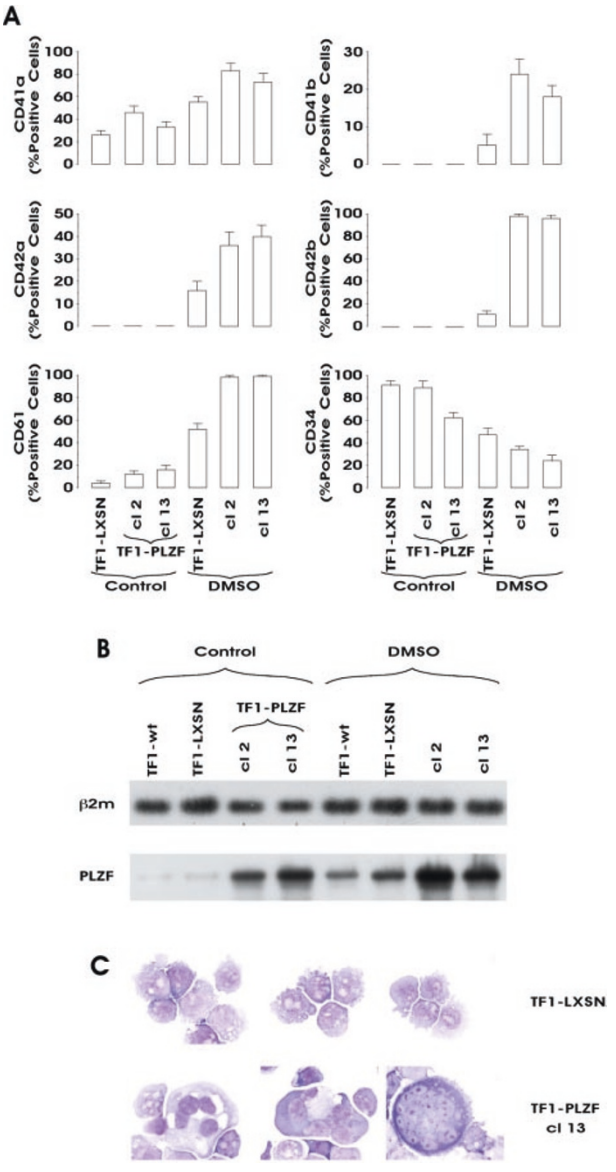

Figure 3 (**a**) Expression of membrane platelet glycoproteins (CD41a, CD41b, CD42a, CD42b, CD61) and CD34 antigen on TF1-PLZF clones cl 2, cl 13 as compared to the control TF1-LXSN cells, grown *in vitro* 4 days either in the absence (control) or in the presence of DMSO. Antigen expression is evaluated by flow cytometry using specific antibodies directly conjugated with fluorochromes. (**b**) RT–PCR analysis of PLZF mRNA expression during DMSO-TF1 cell differentiation. The cell samples are as described above. A representative autoradiogram is shown. Comparable results have been observed in two additional experiments. (**c**) Morphological features of TF1-LXSN and TF1-PLZF cells grown for 4 days in the presence of DMSO. 1000× original magnification. Representative results from three separate experiments are shown

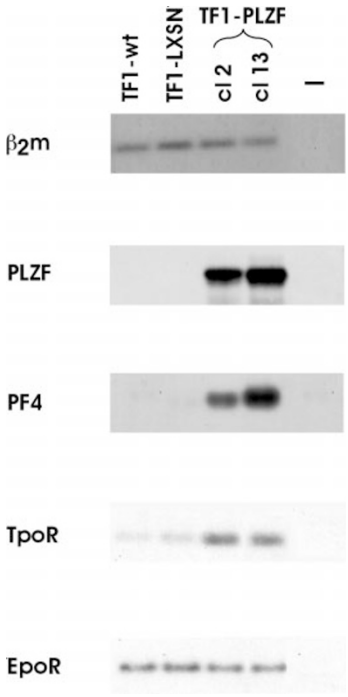

Figure 4 RT–PCR analysis of MK lineage markers in TF1-PLZF cells: mRNA expression of PF4, TpoR and EpoR in: the parental TF1 cells; TF1-LXSN cells; TF1-PLZF cellular clones, cl 2, cl 13. Overexpression of PLZF mRNA is controlled by RT–PCR analysis. β_2 microglobulin was used as an internal control for RT–PCR and to assess the quantity of cDNA used for each sample. The HEL cell line is used as a positive control. A negative control (–) of the RT–PCR reaction is also shown. Representative results from three separate experiments are shown

Interaction of PLZF and GATA1 proteins in TF1-PLZF cells

Northern blot analysis of GATA1 expression in TF1-PLZF cells showed only a slight increase of GATA1 mRNA in both clones, as compared to the control TF1-LXSN (Figure 5a), while GATA1 protein, as assessed by Western blot analysis, was clearly up-regulated in both clones (Figure 5b, Left panel). These observations prompted us to analyse a possible interaction between PLZF and GATA1 proteins. We coimmunoprecipitated PLZF and GATA1 proteins from cell lysates of TF1-PLZF cells (clones 2 and 13) and TF1-LXSN cells (Figure 5c,d). Nuclear protein extracts were immunoprecipitated with either anti-PLZF mAb (Figure 5c) or anti-GATA1 mAb (Figure 5d, Left panel) and loaded onto 9% SDS–PAGE gels for Western blotting analysis. The membranes were probed with anti-GATA1 mAb (Figure 5c), or with anti-PLZF mAb (Figure 5d).

Results showed that the 80 KDa PLZF protein coimmunoprecipitated with the 45 KDa GATA1 endogenous protein in both TF1-PLZF clones (cl 2, cl 13), but not in the control TF1-LXSN cells (Figure 5c,d left panel). The complex between PLZF and GATA1 proteins was observed also after DMSO treatment (data not shown). Similar observations were also made in the erythroid leukemia cell line (HEL) (Figure 5d, right panel), which spontaneously expresses both PLZF and GATA1 proteins at levels detectable by Western blot analysis (Fig. 5b, Right panel).The specificity of PLZF/GATA1 complex was confirmed in control experiments performed by immunoprecipitation of PLZF and pRb105 proteins (Labbaye *et al*., 1999). No PLZF/pRb105 complex was detected in TF1-PLZF cells (data not shown).

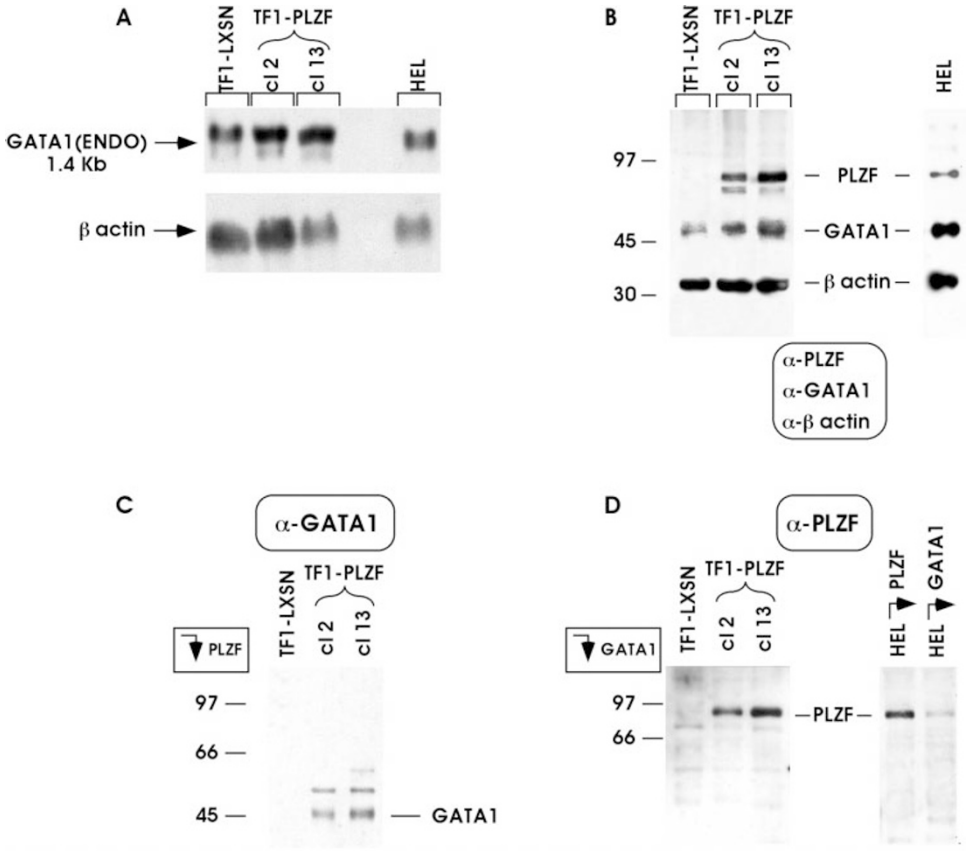

Figure 5 (**a**) Northern Blot analysis of GATA1 mRNA expression in TF1-PLZF cells: Endogenous (ENDO) GATA1 mRNA is around 1.4 Kb and is expressed also in the parental TF1 cells. In TF1-PLZF cells (cl 2, cl 13), GATA1 mRNA is not significantly up-modulated. *β*actin hybridization was performed as a control of RNA normalization. The HEL cell line is used as a positive control. (**b**) Western blot analysis of PLZF and GATA1 proteins expression: the membranes were probed: first with the α-PLZF polyclonal Ab; second with the α-GATA1 mAb; third with the α-*β*actin mAb for the normalization of the proteins in each sample. Endogenous PLZF and GATA1 proteins in HEL cells are shown as a control (right panel). (**c**,**d**) Western blot analysis of nuclear extracts immunoprecipitated with anti-PLZF mAb Figure 5.c: ↓ PLZF) or with anti-GATA1 mAb Figure 5.d: ↓ GATA1) from 2×10^6 cells. The immunoprecipitated materials are analysed by 9% SDS–PAGE. After blotting, the membrane prepared with the anti-PLZF immunoprecipitate was treated with α-GATA1 mAb Figure 5.c: α-GATA1). The membrane prepared with the anti-GATA1 immunoprecipitate is treated with the α-PLZF polyclonal Ab Figure 5.d: α-PLZF, Left panel). Coimmunoprecipitation of endogenous PLZF and GATA1 proteins in HEL cells is shown as a control of the physical interaction of PLZF/GATA1 in these cells (Fig.5D: α-PLZF, right panel). Representative autoradiograms are shown. Molecular weight standards are indicated. GATA1 and PLZF proteins are immunoprecipitated around 45 and 80 KDa, respectively

GATA1 enforced expression in TF1-PLZF cells induces E/MK maturation

To further analyse a possible role for PLZF and GATA1 during MK differentiation of TF1 cells, we cotransduced both PLZF and GATA1 genes in TF1 cells. We used the stably transduced TF1-PLZF cells to overexpress the human GATA1 gene subcloned in the EBV/Retroviral vector Pinco (Pi) (Grignani *et al*., 1998b; Yamaguchi *et al*., 1998). From the TF1-PLZF/GATA1 cotransduced cells, two clones, were selected by flow cytometry to obtain a homogenous population of GFP positive-cotransduced cells.

All controls were prepared and selected for GFP expression by FACS analysis: (i) the TF1 cells stably transduced with the empty Pinco vector, TF1-Pi cells; (ii) the TF1-LXSN cells cotransduced with the Pinco empty vector, TF1-LXSN/Pi cells; (iii) the TF1-PLZF cells cotransduced with the empty vector Pinco, TF1-PLZF/Pi cells; (iv) the TF1 cells stably transduced with only GATA1 full length cDNA subcloned in Pinco vector, TF1-GATA1 cells; (v) the TF1-LXSN cells cotransduced with GATA1 subcloned into Pinco vector, TF1-LXSN/GATA1 cells; (vi) the TF1-PLZF clones 2 and 13 cotransduced with GATA1 subcloned into Pinco, TF1-PLZF/GATA1 cellular clones.

The expression of both PLZF and GATA1 mRNAs in the stably cotransduced cells TF1-PLZF/GATA1 was evaluated by Northern blot analysis (not shown) and RT–PCR analysis (Figure 6). Exogenous GATA1 mRNA (GATA1 EXO) was clearly overexpressed only in TF1-PLZF/GATA1 cells, as compared to the endogenous GATA1 mRNA (GATA1 ENDO) expression present in all subclones of TF1 cells (Figure 6).

By RT–PCR analysis we also evaluated the expression of E (EpoR) and MK (TpoR, PF4) markers in TF1-PLZF/GATA1 cells (Figure 6). We found that the up-regulation of TpoR and PF4 mRNAs observed in TF1-

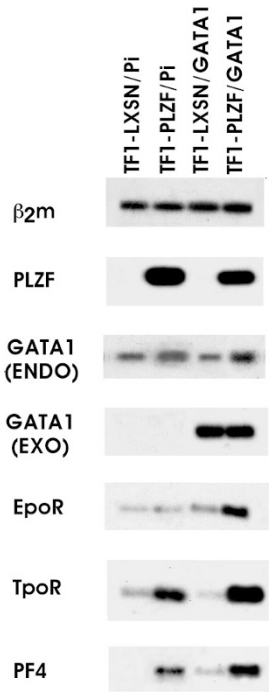

Figure 6 RT–PCR analysis of MK lineage markers in the TF1-PLZF/GATA1 coexpressing cells. EpoR, TpoR and PF4 mRNAs expression is shown in: TF1-LXSN/Pi, the TF1 cells cotransduced with both empty vectors LXSN and Pinco; TF1-PLZF/Pi, the TF1 cells cotransduced with PLZF and the empty Pinco vector; TF1-LXSN/GATA1, the cells cotransduced with the empty vector LXSN and GATA1; TF1-PLZF/GATA1, the TF1 cells cotransduced with both PLZF and GATA1. Overexpression of PLZF, GATA1 endogenous (ENDO) and exogenous (EXO) mRNAs in the cells is controlled by RT–PCR analysis. β_2 microglobulin was used like an internal control. Representative results from three independent experiments are shown

PLZF cells or TF1-PLZF/Pi (Figures 4 and 6) increased when both PLZF and GATA1 genes were coexpressed in the cells (TF1-PLZF/GATA1), while GATA1 alone did not affect the expression of these two MK markers in the control (TF1-LXSN/GATA1) cells (Figure 6). The expression of EpoR mRNA was not affected by the overexpression of PLZF in TF1 cells (Figures 4 and 6), but it is up regulated by the overexpression of GATA1 and, particularly, when both PLZF and GATA1 genes were overexpressed in the TF1 cells (Figure 6).

The analysis of another E marker, glycophorin A, showed that GATA1 overexpression in TF1 cells, but not PLZF overexpression, potentiates the expression of this glycoprotein. TF1-PLZF/GATA1 cells did not express a higher level of glycophorin A as compared to TF1-GATA1 cells (data not shown).

Taken together, our data suggest a cooperation between PLZF and GATA1 to induce the MK differentiation of the TF1 cells.

Transcriptional activation of TpoR promoter in TF1-PLZF cells

To further investigate whether the increase in TpoR expression in TF1-PLZF cells was mediated by a direct effect on the promoter of the TpoR gene, we performed luciferase-based reporter assay using the 1 kb proximal promoter of the TpoR gene that we cloned into the pGL3basic vector, thus obtaining the reporter construct TpoR-FL. This construct was transfected in equal amounts into TF1-LXSN and TF1-PLZF cells, and luciferase activities were assayed 48 h post-transfection. As shown in Figure 8, the TpoR-FL activity was up-regulated and about twofold higher in TF1-PLZF cells than in TF1-LXSN cells. We therefore generated deletion mutants of the TpoR-FL promoter in attempt to identify the region responsible for this effect. Both 5′ and 3′ deletion mutants of the original construct TpoR-FL were prepared (Figure 7a, left), transfected into both TF1-LXSN and TF1-PLZF cells and 48 h later their luciferase activities were measured as compared to the activity detected for the original construct TpoR-FL (Figure 7a, right). The 5′ deletion of the first 500 bp of TpoR-FL (TpoR-Δ3,-Δ4) did not result in a significant decrease of the up-regulation of the TpoR-FL activity (Figure 7a). However, analysing the luciferase activities of 3′ deletion mutants (TpoR-Δ1,-Δ2), cloned into a pGL3-promoter vector substituting the proximal promoter with the SV40 immediate early promoter, we found a significant decrease of the promoter activity as compared to the TpoR-FL activity detected in TF1-PLZF cells (Figure 7a). The luciferase activity of the double 5′ and 3′ deletions mutant was up-regulated in TF1-PLZF cells at levels comparable to those observed in the original construct TpoR-FL (Figure 7a, TpoR-Δ5).

Taken together, our data indicate a functional role for a region of about 250 bp, starting at approximately 500 bp of the transcription start site of the TpoR gene (Figure 7a), whose sequence retains the capability to be activated upon ectopic expression of PLZF in TF1-PLZF cells. Subsequently, we analysed in detail the sequence of this region looking for a putative protein binding site.

Identification of a potential PLZF binding site in the TpoR promoter

The alignment of the different binding sites found for the PLZF protein has allowed to propose a core consensus sequence **A**(T/G)(G/C)**T**(**A**/C)(**A**/C)**AGT** for a potential PLZF DNA-binding site (Li *et al*., 1997) where the TAAAGT sequence seems to be particularly important in the PLZF cognate element.

Analysing the sequence of the 250 bp promoter region identificated in the TpoR-FL (Deveaux *et al*., 1996) we found a potential PLZF-binding site A (site A): 5′-TGAATGGATA**TAAAGT**GCTTAACA-3′. First we have verified that the synthetic PLZF protein can bind to the labeled duplex site A in DNA binding assay (Figure 7b, lane 10). To assess the capacity of the site A to bind nuclear proteins we have performed electrophoretic mobility shift assay (EMSA) using an oligonucleotide containing the site A and nuclear extracts prepared from TF1-LXSN cells (Figure 7b,

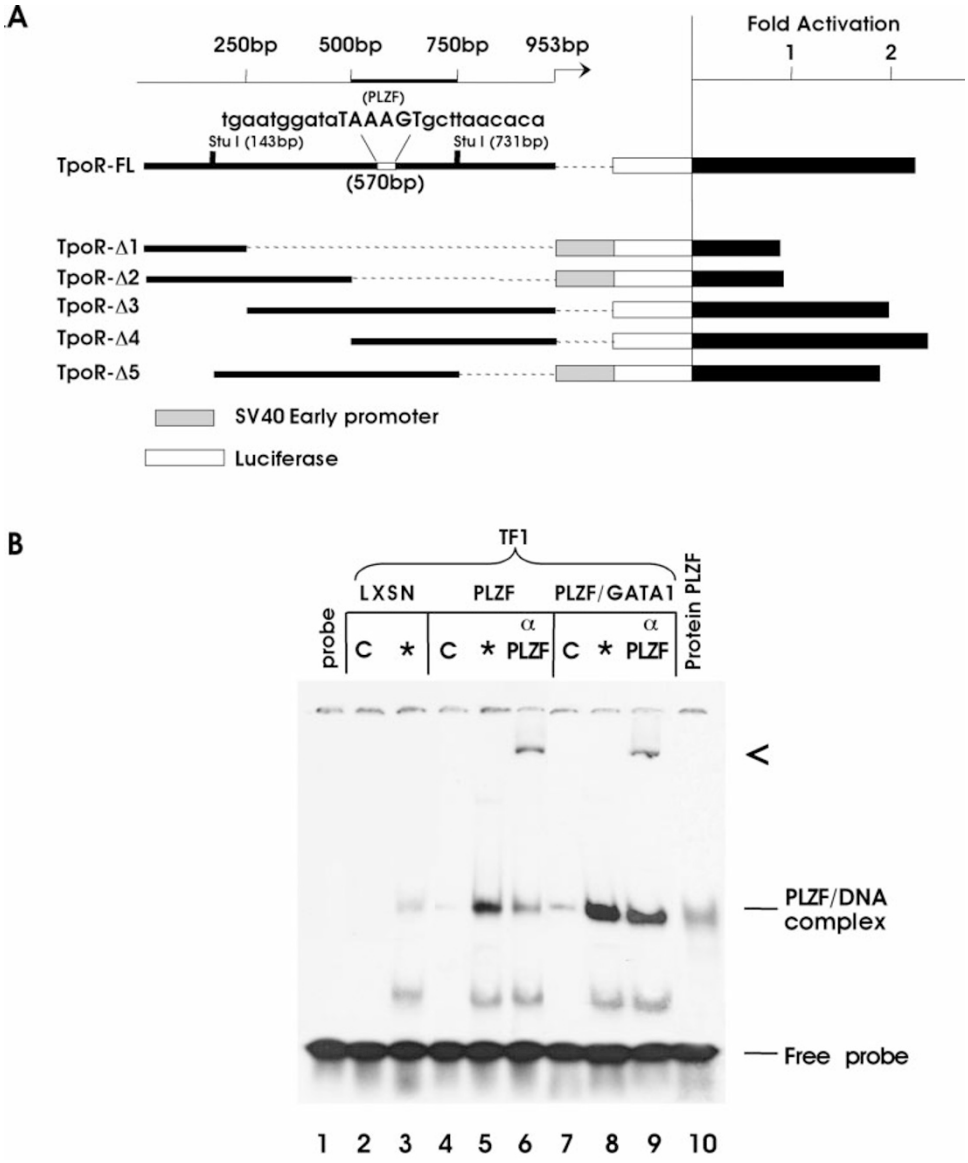

Figure 7 (**a**) The TpoR promoter is activated in TF1-PLZF cells. Left: schematic diagram of the 1 kb fragment of the proximal TpoR promoter (TpoR-FL) and its deletion mutants (TpoR-Δ1,-Δ2,-Δ3,-Δ4,-Δ5). A broken arrow indicates the multiple start sites as described in Mignotte *et al*. (1994) and dashes indicate the deleted regions in the constructs. A putative PLZF DNA-binding site and its surrounding sequence are indicated. Right: Luciferase activity is assayed upon transfection of equal amount of DNA/lipid mixture into equal amounts of cells. The ratio of luciferase activity detected in TF1-PLZF cells respect to TF1-LXSN cells was calculated, resulting in the fold activation shown in the figure. Data are from representative triplicate experiments. (**b**) A PLZF complex binds to the site A, the potential PLZF binding site found in the Tpo receptor promoter. Nuclear extracts from, TF1-LXSN cells, TF1-PLZF cells and TF1-PLZF/GATA1 cells were used in EMSAs. A ^{32}P-labeled oligonucleotide containing the sequence of the Tpo receptor promoter including the site A was used as a probe. The protein-DNA complex was revealed in lanes (*) 5 and 8. Competitive experiments were performed using a 300-fold excess of either the unlabeled oligonucleotide A in lanes (c, competitor) 4 and 7. Supershift experiments were conducted by addition of anti-PLZF polyclonal antibody, lanes (α PLZF) 6 and 9. Lane 10 indicates that the synthetic PLZF protein can bind to the labelled duplex site A. The positions of migration of the free probe (lane 1), the specific PLZF/DNA complexes, and the supershifted complexes are indicated by arrows

lanes 2 and 3), TF1-PLZF cells (Figure 7b, lanes 4, 5, 6), and TF1-PLZF/GATA1 cells (Figure 7b, lanes 7, 8, 9). EMSAs revealed a protein-DNA complex (Figure 7b, lanes 5, 8), which was competed by wild-type oligonucleotide A (Fig.7B, lanes 4, 7), but not by an oligonucleotide in which the PLZF site was mutated (data not shown). Supershift assays using a PLZF polyclonal antibody indicated the presence of PLZF protein in the complex (Figure 7b, lanes 6, 9). In EMSAs carried out with nuclear extracts from TF1-PLZF/GATA1 cells (Figure 7b, lanes 7, 8, 9), the PLZF/DNA complex was always detected (Figure 7b, lane 8) and shifted by the PLZF antibody (Figure 7b, lane 9), thus suggesting that the binding of PLZF protein to the site A was not modified by the overexpression of the GATA1 protein.

Discussion

The development of methods for HPC purification (Gabbianelli *et al*., 1990) and unilineage differentiation/maturation in liquid-phase suspension culture (Labbaye *et al*., 1995, 1999; Guerriero *et al*., 1995; Testa *et al*., 1996) allows a detailed analysis of hematopoiesis through the different lineages at both cellular and molecular level. This model system allowed to investigate the pattern of expression and the possible functional role of the transcription factors GATA1, GATA2, NF-E2, Tal1, PML in hematopoietic differentiation (Labbaye *et al*., 1995, 1999).

Hereby, we applied the unilineage culture system to explore the expression pattern and functional role of PLZF in normal hematopoiesis. PLZF mRNA and protein, expressed in the quiescent HPCs, are downmodulated during both E and G development, while they are moderately upregulated through the MK lineage. This pattern of expression suggests a potential role for PLZF in megakaryopoiesis.

To explore this possible function, PLZF was overexpressed in the human erythroleukemia cell line TF1: the results newly indicate that PLZF stimulates the MK differentiation program and specifically induces TpoR, i.e., the receptor for the pivotal growth factor in MK cell production. Further studies shed light on the molecular mechanism underlying the latter phenomenon: in TF1-PLZF transduced cells, the TpoR proximal promoter was activated and a PLZF-binding site was identified in this DNA region.

Genetic studies demonstrated that GATA1 plays essential roles in E and MK cell differentiation (Pevny *et al*., 1991; Labbaye *et al*., 1995; Shivdasani *et al*., 1997; Takahashi *et al*., 1998; Vyas *et al*., 1999). It seemed of interest to investigate a possible physical and functional interaction between PLZF and GATA1. Coimmunoprecipitation experiments performed in TF1-PLZF transduced cells showed that PLZF formed a complex with GATA1. More important, TF1 cells cotransduced with PLZF and GATA1 showed an enhanced expression of MK and erythroid markers, as compared to cells expressing only either PLZF or GATA1, suggesting a functional significance for PLZF/GATA1 complexes.

In previous reports on leukemic cell lines, such as 32D cells, enforced PLZF expression had an inhibitory effect on G-CSF induced granulocytic maturation, with reduced growth and increased apoptosis (Shaknovich *et al*., 1998; Yang and Korsmeyer, 1996). These findings are in line with our data to suggest that PLZF exerts lineage-specific effects. Thus, overexpression of PLZF in maturing granulocytic cells, in which PLZF is downmodulated, inhibits cell proliferation and maturation. Conversely, overexpression of PLZF in the MK cell pathway, in which the expression of PLZF is up-modulated, stimulates cell maturation without significant growth inhibition.

Recent reports suggested that PLZF acts as an inhibitor of cell growth (Shaknovich *et al*., 1998; Albagli *et al*., 1999). This function was related to the PLZF capacity to form a complex with cdc2 kinase and to inhibit cyclin A expression (Long *et al*., 1998; Ball *et al*., 1999; Yeyati *et al*., 1999). These results are in line with our data. Indeed, PLZF was downmodulated in E and G culture, which are characterized by extensive proliferation (Labbaye *et al*., 1995; Testa *et al*., 1996). Conversely, PLZF expression is initially unaffected and then up-modulated in respectively the early and late stage of MK development, which entails modest proliferation followed by maturation and quiescence of the MK cells (Guerriero *et al*., 1995). Indeed, MK maturation is associated with polyploidy, which occurs after the switch from mitotic to endomitotic cell cycles (Zimmet and Ravid, 2000). The mechanisms determining the polyploidy may involve an inhibition of cyclin A and B, as well as of cdc2 expression/activity (Zimmet and Ravid, 2000). Hypothetically, the increasing expression of PLZF in late megakaryopoiesis may stimulate MK maturation and polyploidy through inhibition of cyclin A/B and cdc2 activity, which may also cause a blockade of cell proliferation. Accordingly, PLZF might coordinately modulate both the growth arrest and the maturation progression in late MK development.

In conclusion, our studies indicate that PLZF plays a significant role in MK development. In this regard, PLZF seemingly functions as a transcriptional modulator of the MK gene program, and specifically induces the Tpo receptor via activation of its proximal promoter. The positive interaction of PLZF and GATA1 in MK development, as well as in erythroid cell production, suggests that PLZF may be involved in the assembly of a multiprotein transcriptional complex that includes GATA1 and possibly other nuclear proteins.

Materials and methods

Hematopoietic growth factors and cell cultures

Recombinant human growth factors were obtained from standard commercial sources. Iscove's medium (IMDM, GIBCO) was freshly prepared weekly.

HPCs were purified from the peripheral blood buffy coat according to a previously reported method (Gabbianelli *et al*., 1990), and successive modifications (Labbaye *et al*., 1995).

The HEL cell line, used as control for Northern blot, RT–PCR analysis and Western blotting/immunoprecipitation experiments, was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS).

The wild-type TF1 cell line as well as the transduced and/or cotransduced TF1 cells were maintained in RPMI medium, supplemented with 10% FCS and 10 ng/ml recombinant human interleukin-3 (IL-3).

HPCs liquid suspension cultures

Unilineage cultures Step IIIP HPCs were seeded (5×10^4 cells/ml) and grown in liquid FCS^- medium supplemented in E culture with low doses of IL-3 (0.001 U/ml) and GM-CSF (0.001 ng/ml) and with a saturating level of Epo (3 U/ml) and in G culture with low amounts of IL-3

(1 U/ml) and GM-CSF (0.1 ng/ml) and with a saturating amount of G-CSF (10 ng/ml) (Labbaye *et al*., 1999). For MK unilineage cultures, HPCs were grown in the presence of Tpo (100 ng/ml) (Guerriero *et al*., 1995). Cells were incubated in a fully humidified atmosphere of 5% CO_2, O_2 atmosphere and were periodically counted and analysed for membrane morphology phenotype, as previously described (Labbaye *et al*., 1999).

Cell surface marker and morphology analysis To evaluate the differentiation of HPCs differentiating along E, G, and MK lineages, the cells were labeled and analysed with a flow cytometer and the cell morphology was performed as previously described (Guerriero *et al*., 1995; Labbaye *et al*., 1999).

Reverse transcriptase (RT-PCR) mRNA analysis of HPCs in unilineages cultures The method for semiquantitative RT–PCR analysis has been previously reported (Labbaye *et al*., 1999). cDNA were normalized with the β_2 microglobulin gene (Labbaye *et al*., 1999). HEL cell line was used as an internal positive control. An aliquot of RNA (20 ng) from each sample and a mock reaction (negative control) were amplified to exclude the presence of contaminant DNA.

PLZF primers and probe were as follows: PLZF sense1, 5′-(ATgATCCAgCTgCAgAAC)-3′ from 16 to 33; PLZF α-sense1, 5′-(CCggCTCTCTgACTTCAT)-3′ from 1182 to 1200; internal probe 1, 5′-(TgCAgTggACAgTTTgATgACCATAg-gACAg)-3′ from 603 to 634 (Chen *et al*., 1993b). The amplification procedure included denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s. during 30 PCR cycles. In control experiments, serial dilutions of samples were amplified; the dose-response curves showed linearity for all points, indicating that the amplification procedure was within the range of linear cDNA dose response (not shown). Relative intensities of bands were quantified by scanning with a laser densitometer (Phosphorimager Molecular Dynamics Inc, Sunnyvale, CA, USA).

Gene transfer

Enforced expression of PLZF cDNA in TF1 cells RT–PCR was performed to prepare the human full length PLZF cDNA from the HEL PLZF-positive cell line. The PCR reaction was set-up using the 18 bp sense primer sequence: 5′-(ATggATCTgACAAAAATg)-3′ from 76 to 93 bp and the antisense primer used was a sequence from 2080 to 2097 bp 5′-(TCACACATAgCACAggTA)-3′ of the published sequence (Chen *et al*., 1993b). The human full length PLZF cDNA controlled by automated DNA sequencing was then subcloned in the LXSN retroviral vector (Valtieri *et al*., 1994). The construct LXSN-PLZF is referred to as (PLZF). LXSN vector, lacking the PLZF cDNA, was used as mock control virus. The retroviral packaging cell line was prepared as described in Condorelli *et al*. (1997). The TF1 cell line was transduced using the method described in Kotani *et al*. (1994). TF1-PLZF cells subcloning was performed and the single clones were grown in 0.1 ml of RPMI complete medium supplemented with 0.8 mg of Geneticin per ml. Cells from each clone were smeared onto glass slides by centrifugation and analysed for PLZF expression by immunofluorescence using an anti-PLZF monoclonal antibody (Shaknovich *et al*., 1998). Two cellular clones TF1-PLZF (cl 2, cl 13), displaying the highest positivity (80 and 95% strongly positive PLZF cells, respectively) were maintained in complete medium with 0.4 mg of Geneticin per ml and utilized for functional studies.

Enforced expression of GATA1 cDNA in TF1-PLZF cells To transduce the TF1-PLZF cells with the GATA1 gene, we have used the full-length cDNA for human GATA1 subcloned into the recently described retroviral vector Pinco-GFP to produce the viral supernatant from the retroviral packaging cell line, Phoenix, as described in Grignani *et al*. (1998b). We have transduced GATA1 (i) in the TF1-wt cells, TF1-GATA1; (ii) in the TF1-LXSN cells, TF1-LXSN/GATA1; (iii) in both clones 2 and 13 of TF1-PLZF cells, TF1-PLZF/GATA1. Two stably cotransduced cells, TF1-PLZF/GATA1, were selected for high levels of Green Fluorescence Protein (GFP) expression detected by FACS analysis and cultured in RPMI medium supplemented with 10% FCS and IL-3.

Northern blot analysis

Total RNA or polyA$^+$ mRNA were prepared (Dynabeads mRNA purification kit, Dynal) from TF1 cells and their subclones for Northern blot analysis according to standard procedures. The filters were hybridized with a labeled cDNA probes: PLZF ~1.9 Kb; GATA1 ~1.2 Kb; β-actin ~1.9 Kb used for the normalization of the samples.

Immunofluorescence analysis

TF1, TF1-LXSN, TF1-PLZF cells were smeared onto glass slides by cytospin centrifugation. Procedure was performed as previously described (Labbaye *et al*., 1999). The anti-PLZF mAb used for immunofluorescence labeling of PLZF was described (Shaknovich *et al*., 1998).

Cell surface markers

To evaluate the differentiation status of TF1 cells transduced or not with PLZF, the reactivity of the cells with different monoclonal antibodies directly conjugated with fluorochromes was evaluated. TF1 cells were incubated with different types of monoclonal antibodies as follows: anti-MK membrane antigens (CD41, CD41a, CD41b, CD42b, CD61, CD62); anti-E membrane antigens (glycophorin A); anti-progenitor cell antigens (CD34). All these antibodies were obtained from Pharmingen. Labeled cells were analysed for fluorescence emission using a flow cytometer (FACSCAN, Becton Dickinson, Mountain View, CA, USA).

DMSO treatment of TF1 (PLZF) cells

Cells were incubated for 4 days in the presence of 0.8% DMSO (Sigma, St. Louis, USA) and then, either analysed for differentiation markers expression by immunofluorescence as described above, or analysed for PLZF expression by RT–PCR as described above. Cells were cytospin on glass slides and stained with May-Grünwald for morphological analysis.

RT–PCR analysis in transduced and cotransduced cells

Quantitative RT–PCR analyses were performed according the procedures described in Labbaye *et al*., 1999. PCR products for each samples were analysed by Southern blot and hybridized with an internal oligomer probe (Labbaye *et al*., 1999).

To evaluate the expression of GATA1 (Wong and Privalsky, 1998), PF4 (Ravid *et al*., 1991), TpoR (Vignon *et al*., 1992), and EpoR (Maouche *et al*., 1991) genes, the sequences of primers and probes and the PCR conditions were as follows. Primers included 5′(TTAgCCACCT-

CATgCCTT)3′ and 5′(gAgACTTgggTTgTCCAg)3′ for GATA1 endogenous (endo); 5′(TTAgCCACCTCATgCCTT)3′ and a primer from the specific sequence of Pinco vector cDNA 5′(CCCgATAC-TTgATTACTg)3′ for GATA1 exogenous (exo); 5′(gCgCTgA-AggTgAAgAAgATg)3′ and 5′(gCACACACgTAggCAgCT-Agt)3′ for PF4; 5′(AgCTgATTgCCACAgAAACC)3′ and 5′(ACTTggggAggTCTgCTTTg)3′ for TpoR; 5′(TCATggACCACCTCggggCgT)3′ and 5′(TAgCggATgTgAgACgTCA-Tg)3′ for EpoR. Internal probes included 5′(gTggTggCTCC-gCTCAgCTCATg) for GATA1; 5′(TCACCAgCCTggAggTgATCAAggC)3′ for PF4; 5′(CCAgTCTCCATgTgCTCAgC-CCACAATgCC)3′ for TpoR; 5′-(TCTggTgTTCgCTgCCTACAgCCgACACgTC)3′ for EpoR. PCR conditions were (95°/30 s, 54°/30 s, 72°/45 s) for both GATA1 endo and exo; (95°/30 s, 58°/30 s, 72°/45 s) for PF4; (95°/30 s, 60°/30 s, 72°/45 s) for TpoR; (95°/30 s, 56°/30 s, 72°/45 s) for EpoR. All control experiments were performed according to Labbaye *et al*. (1995, 1999).

Immunoprecipitation and Western blot analysis

Nuclear extracts were prepared from TF1 and TF1-PLZF cells (cl 2, cl 13) and immunoprecipitated as described in Labbaye *et al*. (1999), with (i) anti-PLZF mAb (Shaknovich *et al*., 1998) diluted 1 : 1000; (ii) anti-h GATA1 mAb (GATA1(N1) sc-266, Santa Cruz) diluted 1 : 500; (iii) anti-β actin mAb (Calbiochem, San Diego, CA, USA) diluted 1 : 10 000 to quantify the proteins loaded for each sample.

Immune complex samples were prepared in duplicate and loaded onto two different 9% SDS–polyacrylamide gels. Blotted membranes were probed: (i) with anti-PLZF polyclonal antibody (Melnick and Licht, 1999), diluted 1 : 5000 or with the anti-hGATA1 mAb (Santa Cruz), diluted 1 : 500; (ii) the membrane first treated with anti-PLZF, was stripped and reprobed with anti-hGATA1 mAb, and the membrane first treated with the anti-hGATA1 was stripped and reprobed with anti-PLZF polyclonal antibody. As a control, the filters were stripped and reprobed with the same antibody used for immunoprecipitation, confirming the presence of the GATA1 protein in anti-GATA1 immunoprecipitates, or the presence of the PLZF protein in anti-PLZF immunoprecipitates. Membranes were visualized by Enhanced Chemiluminescence (Amersham).

Luciferase assay

The proximal promoter region of the human TpoR gene (Mignotte *et al*., 1994) was amplified by PCR on Hela genomic DNA using primers based on the nucleotide sequence deposited as Genebank U68159. The PCR product with synthetic cutting sites for *Xho*I and *Bgl*II was subcloned into the luciferase reporter vector pGL3 basic (Promega), yielding p-TpoR-GL3 (TpoR-FL). This latter construct was used as a template for PCR with oligonucleotides harbouring 5′ and 3′ deletion derivatives as shown in Figure 7, and the products were subcloned in the *Xho*I and *Bgl*II sites of pGL3 basic vector (TpoR-Δ3,-Δ4) or pGL3Promoter vector (TpoR-Δ1,-Δ2) (Promega). For the mutant TpoR-Δ5, a *Stu*I fragment of TpoR-FL was cloned blunt into a pbluescript KS+ vector and digested with *Bam*HI and *Xho*I to be cloned into the *Bgl*II and *Xho*I sites of the pGL3Promoter vector. Details of the primers sequences are available upon request. All the constructs were verified by sequencing. TF1-LXSN cells used as a control and TF1-PLZF cells, were transfected with FUGENE 6 (Roche) using a lipid (μl)/DNA (μg) ratio of 3. The lipid-DNA complexes were incubated with the cells (1×10^5) in serum free medium 4 to 6 h before adding serum to the cultures. Cell lysates were prepared 48 h after transfection and assayed for luciferase activity using the Dual Luciferase Reporter System Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, luciferase activity was measured in a TLX luminometer, using pRL-SV40 (Promega, Madison, WI, USA) for the normalization of luciferase activities. Data are presented as the ratio between the relative luciferase activity detected in TF1-PLZF cells as compared to TF1-LXSN cells. The luciferase activities presented reflect triplicate values and similar results were obtained from at least three independent experiments.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed according the procedure described in Li *et al*. (1997). Each binding reaction (20 μl) contained, 5 μg of nuclear extracts in a buffer of 20 mM ZnCl, 10 mM Tris-HCl, pH 7.5, 10 mM MgCl, 50 mM NaCl, 1 mM dithiothreitol and the ^{32}P-labeled double-strand oligonucleotide probe A: 5′-TGAATGGATATAAAGTGCTTAACA-3′ (Deveaux *et al*., 1996). After 45 min on ice, the protein-DNA complexes were resolved on a 5% polyacrylamide gel and visualized by autoradiography. A 300-fold molar excess of unlabeled oligonucleotide probe A or unlabeled oligonucleotide mutated, mt: 5′-TGAACGGGCACACCTCGCTTAACA-3′ was used for competition experiments and 2 μl of α PLZF polyclonal antibody was used for supershift assay.

Acknowledgments
We thank M Blasi and M Fontana for editorial assistance and A Zito and G Loreto for graphics. This work was partially supported by NIH grant CA 59936 to JD Licht.

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