TLS-ERG Leukemia Fusion Protein Deregulates Cyclin-Dependent Kinase 1 and Blocks Terminal Differentiation of Myeloid Progenitor Cells

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Abstract

TLS-ERG fusion protein is derived from the t(16;21) translocation found in human myeloid leukemia. Here, we show that retroviral transduction of TLS-ERG confers a growth advantage to L-G myeloid progenitor cells and blocks terminal differentiation. We found that the level of cyclin-dependent kinase 1 (Cdk1) protein was significantly decreased in controls but unchanged in TLS-ERG–expressing cells after granulocyte colony-stimulating factor treatment or interleukin-3 withdrawal. Injection of TLS-ERG–expressing L-G cells induced rapid development of a leukemia-like disease in syngeneic mice. Through site-directed mutagenesis, we showed that transformation and deregulation of Cdk1 by TLS-ERG require an intact *ets* DNA-binding domain within the fusion protein. Interestingly, treatment of TLS-ERG–expressing L-G cells with 5-aza-2′-deoxycytidine (Decitabine) or trichostatin A resulted in down-regulation of Cdk1 and induction of terminal differentiation. To investigate whether Cdk1 deregulation is indeed responsible for transformation by TLS-ERG, we constructed lentiviral vectors for delivery of Cdk1 mutants and small interfering RNA (siRNA). Both dominant-negative inhibition and siRNA knockdown of Cdk1 were able to restore the ability of TLS-ERG–expressing L-G cells to undergo terminal differentiation. In addition, siRNA knockdown of Cdk1 in YNH-1 cells derived from a t(16;21) acute myelogenous leukemia patient also resulted in terminal differentiation. As restoration of terminal myeloid differentiation to TLS-ERG cells is dependent on cell cycle arrest, our findings suggest an important role for Cdk1 in cellular transformation and may be useful in the search for new treatments of TLS-ERG–associated myeloid leukemia. (Mol Cancer Res 2008;6(5):862–72)

Introduction

Since the discovery of the Philadelphia chromosome in chronic myeloid leukemia, a large number of leukemias have been found to be associated with specific chromosomal translocations. In a subset of acute myelogenous leukemia, the NH_2-terminal region of TLS (translocation liposarcoma) is fused to the COOH-terminal domain of ERG (*ets*-related gene) through a recurrent t(16;21) translocation (1). The resultant TLS-ERG fusion protein is associated with poor clinical outcome (2). In an earlier report, retroviral transduction of $CD34^+$ human hematopoietic cells from cord blood showed that TLS-ERG was able to block erythroid differentiation and increase the proliferation and self-renewal of myeloid progenitors (3). More recently, retroviral transduction of TLS-ERG in primitive human hematopoietic cells revealed additional cooperating genetic events, such as up-regulation of the polycomb group gene *Bmi-1*, the telomerase gene *hTERT*, and the appearance of trisomy 10 and 19 during prolonged culture (4).

Although TLS-ERG fusion is the primary genetic event leading to cellular transformation in acute myelogenous leukemia patients harboring t(16,21) translocation, how this fusion protein alters the differentiation, proliferation, and self-renewal of hematopoietic cells remains unclear. TLS-ERG has been reported to affect both gene transcription and RNA splicing by several groups in fibroblasts and in hematopoietic cells (5, 6). Our recent studies further suggested that transformation of L-G myeloid progenitor cells by TLS-ERG is likely to be associated with a repressive effect of the fusion protein on gene transcription (7).

Leukemia cells are characterized by an advantage in proliferation and an arrest of differentiation at various stages of hematopoiesis. Studies of myeloid development have revealed that cell cycle status is closely associated with functions of the cells at each step within the differentiation pathway: Slow cell cycle or dormancy is necessary for self-renewal of primitive stem cells; rapid cell cycle is required for effective expansion of the progenitor population; and withdrawal from the cell cycle is a prerequisite for various functions of terminally differentiated cells. For hematopoietic progenitors

to undergo terminal differentiation, they have to exit the cell cycle at some point along the differentiation pathway. For granulopoiesis, myeloid progenitors go through distinct stages that are morphologically characterized as myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells, and segmented neutrophils. Myelocytes are the last stage to undergo mitosis, and cells in subsequent stages continue to mature but do not divide (8).

In this study, we have investigated whether TLS-ERG transforms L-G cells through deregulation of cell cycle molecules. Our results indicate that although the protein level for cyclin-dependent kinase 1 (Cdk1) decreases during granulocyte colony-stimulating factor (G-CSF)–induced terminal differentiation or after interleukin 3 (IL-3) withdrawal in L-G cells harboring the empty retroviral vector, Cdk1 level is not affected under similar conditions in TLS-ERG–expressing L-G cells. Inhibition of Cdk1 activity through a dominant-negative mutant or small interfering RNA (siRNA) knockdown unlocks TLS-ERG blockage of terminal differentiation. These findings suggest that TLS-ERG leukemia fusion protein transforms myeloid progenitor cells through deregulation of Cdk1, thus escaping from control by the cell cycle machinery.

Results

TLS-ERG Blocks Terminal Differentiation of L-G Myeloid Progenitor Cells

Mouse L-G myeloid progenitor cells are dependent on IL-3 for proliferation and undergo terminal differentiation into mature neutrophils when treated with G-CSF. To investigate the effect of TLS-ERG on the growth and differentiation of L-G cells, we constructed a retroviral vector for stable expression of Flag-tagged TLS-ERG fusion protein. After transduction of L-G cells with an empty retrovirus or a retrovirus containing TLS-ERG cDNA, the cells were selected with G418 and expression of the fusion protein was confirmed by Western blotting (Fig. 1A).

Like the parental cells, L-G cells harboring the empty retroviral vector stopped proliferation in G-CSF medium, whereas cells expressing TLS-ERG continued to grow (Fig. 1B). G-CSF–treated L-G cells harboring the empty vector eventually differentiated into morphologically recognizable neutrophils whereas cells expressing TLS-ERG did not change in morphology (Fig. 1C). As cells undergoing terminal differentiation have to exit the cell cycle, we carried out Western blotting to examine expression of Cdk1 and Cdk2, two important cell cycle regulators. After 3 days of treatment in G-CSF, Cdk1 was found
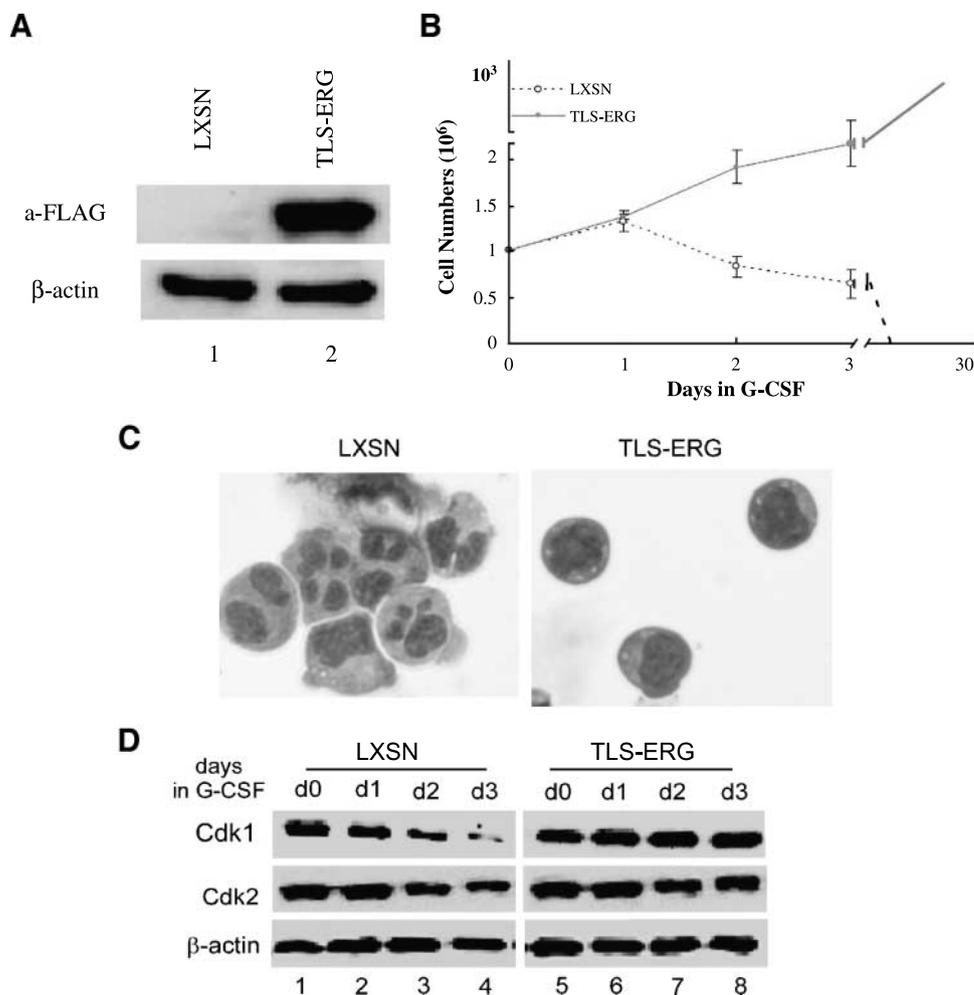
FIGURE 1. Blockage of terminal differentiation and deregulation of Cdk1 by TLS-ERG in L-G cells. **A.** Nuclear extract was prepared from L-G cells harboring the empty retroviral vector (*lane 1*) or those expressing Flag-TLS-ERG (*lane 2*) for Western blotting with the M2 anti-Flag antibody. **B.** IL-3 was removed from the medium followed by addition of G-CSF to 10 ng/mL. Cell growth was monitored daily and the numbers of viable cells are shown as averages of multiple counts. **C.** Seven days after G-CSF treatment, L-G cells were collected for Wright-Giemsa staining. Lobulated nuclear morphology of mature neutrophils was more abundant in cells harboring the empty retroviral vector LXSN. **D.** Whole-cell lysates were prepared from L-G cells harboring the empty retroviral vector (*lanes 1-4*) or those expressing TLS-ERG (*lanes 5-8*) at the indicated time points after treatment with G-CSF. The samples were separated by SDS-PAGE and blotted with specific antibodies.
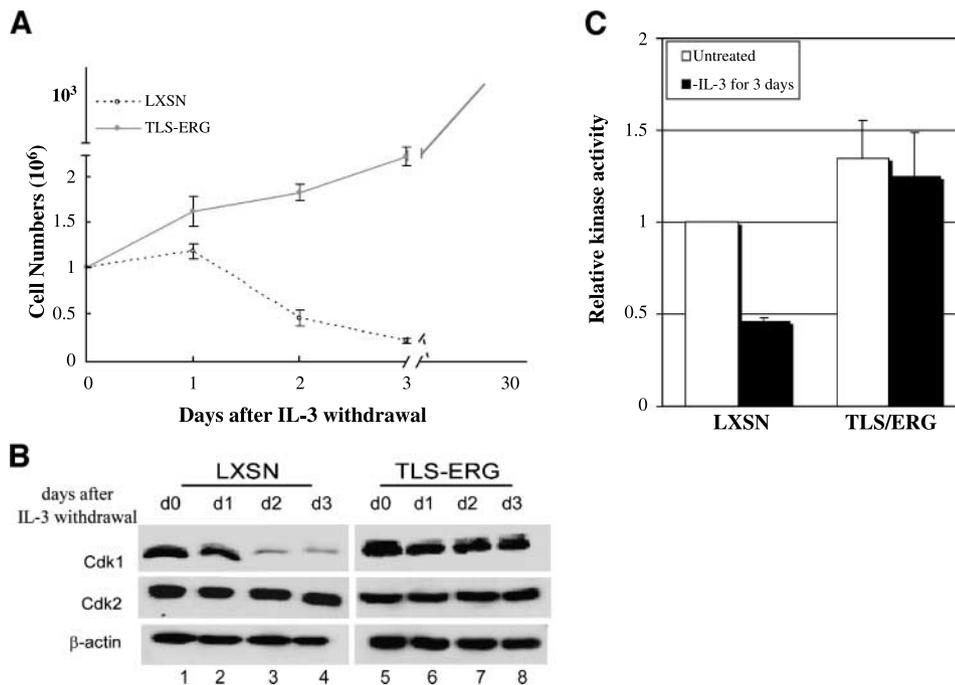
FIGURE 2. Abrogation of IL-3 requirement and deregulation of Cdk1 by TLS-ERG in L-G cells. **A.** IL-3 was removed from the medium by extensive washing with PBS. Cell growth in medium without IL-3 was monitored daily and the numbers of viable cells are shown as averages of multiple counts. **B.** Whole-cell lysates were prepared from L-G cells harboring the empty retroviral vector (*lanes 1-4*) or those expressing TLS-ERG (*lanes 5-8*) at the indicated time points after IL-3 withdrawal for Western blot analysis with specific antibodies. **C.** Cdk1 kinase assays were done with lysates from L-G cells harboring the empty retroviral vector (*columns 1-2*) or those expressing TLS-ERG (*columns 3-4*) before and after IL-3 withdrawal. Results are from three independent experiments.

to be significantly down-regulated in L-G cells harboring the empty vector. In cells expressing TLS-ERG, however, Cdk1 level was not affected by G-CSF treatment (Fig. 1D). In contrast to Cdk1, the protein level of Cdk2 was not significantly affected by G-CSF treatment in either cell population, suggesting that Cdk2 is not involved in TLS-ERG blockage of terminal differentiation in L-G myeloid progenitor cells.

TLS-ERG Abrogates IL-3 Dependency

As TLS-ERG expression in L-G myeloid progenitor cells was able to block G-CSF–induced terminal differentiation, we next investigated the possibility that these cells have acquired an ability to grow spontaneously and no longer need IL-3 for proliferation. In this experiment, IL-3 was removed from the culture medium by extensive washing with PBS, and the cells were then placed in medium without IL-3. Three days after IL-3 withdrawal, we observed that L-G cells harboring the empty vector had decreased to about one fourth of the original number, whereas those expressing TLS-ERG kept growing and more than doubled in number (Fig. 2A). When examined by Western blotting, IL-3 withdrawal significantly decreased Cdk1 protein level in L-G cells harboring the empty vector but had minimal effect on Cdk1 protein in cells expressing TLS-ERG. Similar to G-CSF treatment, IL-3 withdrawal did not affect Cdk2 protein in either cell population (Fig. 2B). The Cdk1 kinase activities in these cells were also assayed following IL-3 withdrawal. Although Cdk1 activity was dependent on IL-3 in the culture medium for LXSN controls, TLS-ERG–expressing cells appeared to maintain a robust Cdk1 activity that is less dependent on IL-3 (Fig. 2C).

TLS-ERG Induces a Leukemia-Like Disease in Mice

We have shown that expression of the TLS-ERG fusion protein enabled L-G myeloid cells to proliferate without IL-3

and made them resistant to G-CSF–induced terminal differentiation. As these experiments were carried out in cell culture, they may represent an *in vitro* artifact that did not truly reflect *in vivo* situations. To investigate whether TLS-ERG induces a leukemia-like disease *in vivo*, L-G cells harboring the LXSN retroviral vector or expressing TLS-ERG fusion protein were i.v. injected into the syngeneic BALB/c mice. After 6 weeks, none of the 8 animals injected with LXSN cells developed obvious disease. In comparison, 4 of 8 mice injected with TLS-ERG cells developed a disease resembling leukemia with symptoms such as splenomegaly (Fig. 3A), enlargement of the liver, and infiltration of the bone marrow by blast-like cells (data not shown). When spleen sections from the control and diseased animals were analyzed, it became clear that normal histologic features of the spleen were disrupted and the presence of myeloid cells were prominent in mice injected with TLS-ERG–expressing L-G cells (Fig. 3B).

Transformation and Deregulation of Cdk1 by TLS-ERG Require an Intact *ets* Domain

How did TLS-ERG fusion protein deregulate Cdk1 expression and transform L-G myeloid progenitor cells? We next performed experiments to further understand the potential mechanisms of transformation caused by this fusion protein. TLS-ERG contains an intact *ets* DNA-binding domain and is capable of binding to DNA in a sequence-specific manner (5). A point mutation within the *ets* domain that replaces the evolutionarily conserved arginine at position 367 with leucine (R367L) has been known to abolish DNA binding (9, 10). To determine whether transformation by TLS-ERG requires an intact *ets* domain, we stably expressed TLS-ERG (R367L) mutant in L-G cells by retroviral transduction, then subjected these cells to G-CSF treatment or IL-3 withdrawal. Unlike cells expressing TLS-ERG, cells expressing TLS-ERG(R367L)

decreased to about half of the original number 3 days after G-CSF treatment and started to exhibit morphology of terminally differentiated neutrophils (Fig. 4A, *left*, and data not shown). R367L point mutation also abolished the ability of TLS-ERG cells to grow without IL-3 (Fig. 4A, *right*). In addition, this R367L mutant also failed to maintain the level of Cdk1 protein following G-CSF treatment or IL-3 withdrawal (Fig. 4B). These results showed that transformation and deregulation of Cdk1 protein by TLS-ERG require a functional *ets* DNA-binding domain within the fusion protein.

Epigenetic Drugs Down-Regulate Cdk1 and Promote Differentiation in TLS-ERG Cells

Our recent studies have suggested that TLS-ERG transformation of L-G myeloid progenitor cells is likely associated with a repressive effect of the fusion protein on gene transcription (7). Silencing of genes critical to myeloid differentiation is a

FIGURE 3. Development of a leukemia-like disease. **A.** Six weeks after injection, spleens from two mice inoculated with L-G cells harboring the LXSN vector were compared with those from two mice inoculated with TLS-ERG–expressing L-G cells (average weight 83 ± 8 mg versus 884 ± 91 mg). **B.** Gross histologic differences between spleen sections from the animal injected with L-G cells harboring the LXSN vector (*left*) and from the one injected with TLS-ERG–expressing L-G cells (*right*) are evident after staining with H&E. Arrow indicates a rectangle box that is shown at a higher magnification below. Bar, 50 µm.
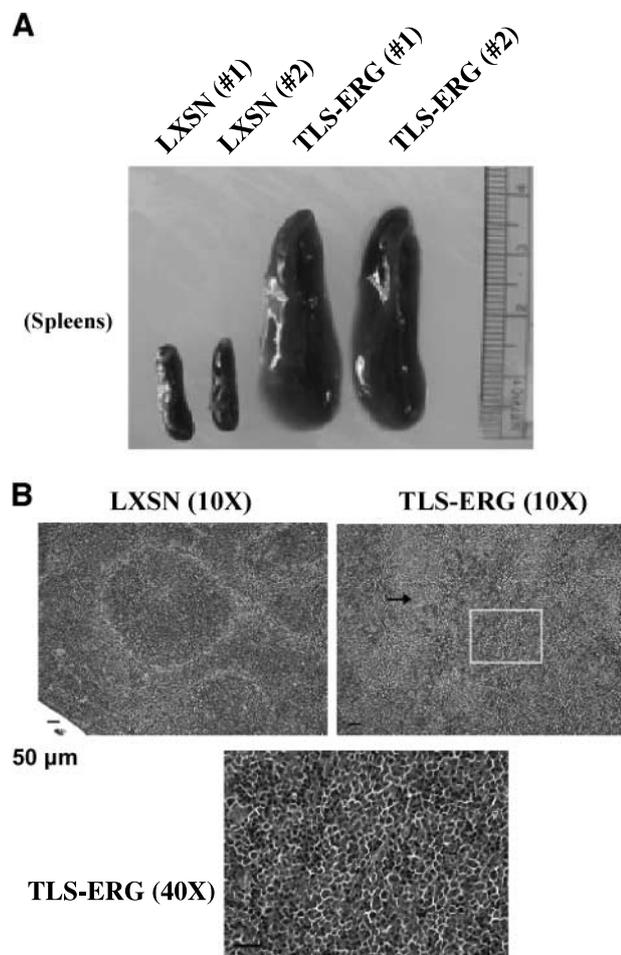
well-known phenomenon associated with leukemia fusion proteins. The most notable case involves acute promyelocytic leukemia in which a t(15;17) translocation fuses PML protein with the retinoic acid receptor-α, and gene repression by the resultant PML-RARα can be reversed by treatment with *all-trans* retinoic acid (11).

As gene repression by TLS-ERG might be mediated through recruitment of repressor complexes involving DNA methylation and histone deacetylation, TLS-ERG–transformed L-G cells were treated with 5-aza-2′-deoxycytidine (also called Decitabine, a DNA methyltransferase inhibitor) and trichostatin A (a histone deacetylases inhibitor). When compared with untreated TLS-ERG cells in G-CSF medium, 5-aza-2′-deoxycytidine or trichostatin A was able to slow down cell growth and restore the ability of these cells to undergo terminal differentiation into mature neutrophils (Fig. 5A). When examined by Western blot analysis, Cdk1 protein level was significantly decreased in TLS-ERG cells following treatment with 5-aza-2′-deoxycytidine or trichostatin A (Fig. 5B). In control L-G cells harboring the empty LXSN retroviral vector, treatment with these two inhibitors also accelerated down-regulation of Cdk1 (Fig. 5C), suggesting that the effects of these molecules are not specific to TLS-ERG cells.
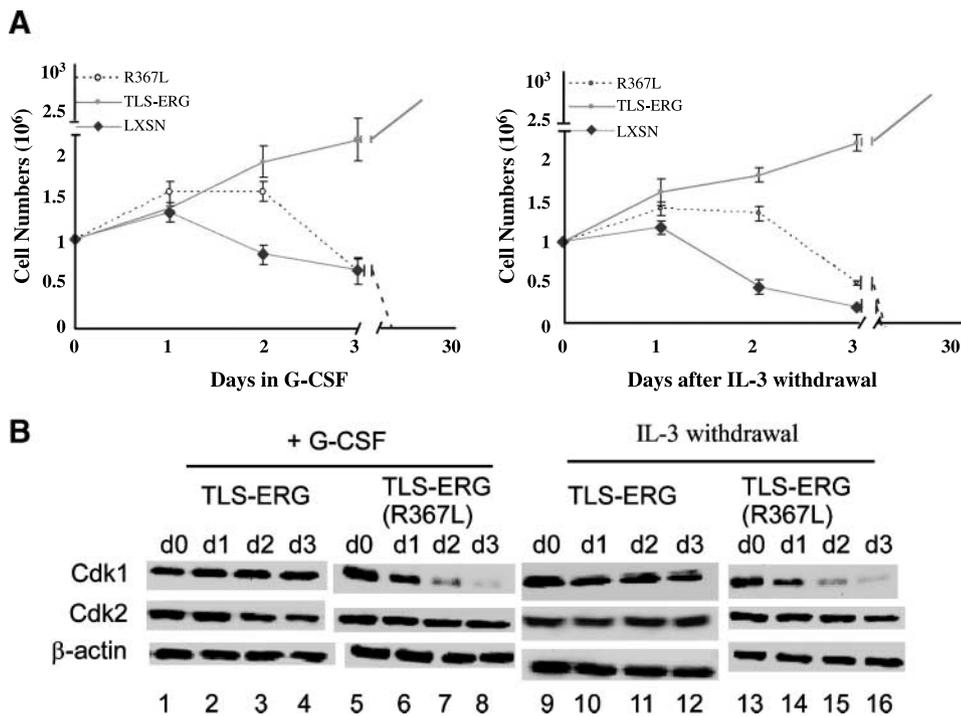
Dominant-Negative Inhibition of Cdk1 Unlocks Differentiation Blockage by TLS-ERG

Although restoration of terminal differentiation in TLS-ERG cells by 5-aza-2′-deoxycytidine or trichostatin A is accompanied by a decrease in Cdk1 protein, the pleiotropic effects of these epigenetic drugs made it difficult to conclude that down-regulation of Cdk1 was responsible for the observed phenotype change in the absence of additional supporting data.

The kinase activity of Cdk1 is regulated by phosphorylation on several critical amino acids: Simultaneous mutations of T14A and Y15F result in a constitutively active Cdk1 (Cdk1-AF; ref. 12) and D145N point mutation generates a dominant-negative, kinase-dead Cdk1 (Cdk1-DN; ref. 13). Because Cdk1 kinase activity might be critical for differentiation blockage by TLS-ERG fusion protein, we used the dominant-negative form of Cdk1 (D145N) to investigate whether specific inhibition of Cdk1 kinase activity had any effect on differentiation of TLS-ERG cells. To this end, Flag epitope–tagged Cdk1-WT, Cdk1-AF, and Cdk1-DN were cloned into a modified pLL3.7 lentiviral vector that coexpresses green fluorescence protein (GFP; ref. 14). After lentiviral infection of TLS-ERG cells, GFP-positive cells were enriched by sorting on a flow cytometer and were used for protein analysis. When blotted with an anti-Flag antibody, the epitope-tagged Cdk1 mutants were indeed expressed in these GFP[+] TLS-ERG cells (Fig. 6A, *top*). To rule out the possibility that any potential effect could be due to protein overexpression, the same lysates were also blotted with an anti-Cdk1 antibody and the amount of exogenous Cdk1 was shown to be slightly less than endogenous Cdk1 (Fig. 6A, *second panel*). After treatment with G-CSF, TLS-ERG cells transduced with the empty lentiviral vector, or harboring Cdk1-WT and Cdk1-AF, only had less than 10% of mature neutrophils (band and segmented cells); however, Cdk1-DN expression in TLS-ERG cells resulted in a 3-fold

FIGURE 4. Requirement for an intact *ets* domain in cellular transformation and deregulation of Cdk1 by TLS-ERG. **A.** LG cells harboring the LXSN vector or expressing TLS-ERG and the R367L mutant were treated with 10 ng/mL G-CSF (*left*) or cultured in medium without IL-3 (*right*). Cell growth was monitored daily and the number of viable cells is shown as averages of multiple counts. **B.** Whole-cell lysates were prepared from L-G cells expressing TLS-ERG or the R367L mutant at the indicated time points after G-CSF treatment (*lanes 1-8*) or following IL-3 withdrawal (*lanes 9-16*), and the lysates were blotted with specific antibodies.

increase of mature neutrophils (band and segmented cells; Fig. 6B). Thus, it appears that dominant-negative inhibition of Cdk1 kinase activity is sufficient to unlock blockage of terminal differentiation by TLS-ERG fusion protein in L-G cells.

siRNA Knockdown of Cdk1 Forces Terminal Differentiation of TLS-ERG Cells

To further confirm that Cdk1 is indispensable for differentiation blockage by TLS-ERG in L-G cells, we carried out experiments to knockdown Cdk1 in TLS-ERG cells by RNA interference. For this purpose, siRNA-targeting mouse Cdk1 was designed and cloned into the pLL3.7 lentiviral vector, in which GFP is coexpressed and transcription of the short hairpin siRNA is driven by the U6 promoter. After lentiviral infection of TLS-ERG cells, the GFP+ fraction was enriched by flow cytometry sorting and used for Western blotting analysis. TLS-ERG cells harboring the empty lentiviral vector or expressing a scrambled siRNA had no change in Cdk1 protein level, whereas cells harboring Cdk1 siRNA had a dramatic reduction in Cdk1 protein level (Fig. 7A, *top*). The siRNA knockdown of Cdk1 appeared to be specific as Cdk2 or β-actin level was not affected by the siRNA (Fig. 7A, *middle* and *bottom*). To investigate the morphologic effect of siRNA knockdown of Cdk1, GFP+ cells were treated with G-CSF and examined by Wright-Giemsa staining. Whereas TLS-ERG cells harboring the empty lentiviral vector or expressing scrambled siRNA maintained a morphology similar to that of the uninfected TLS-ERG cells, siRNA knockdown of Cdk1 protein increased the percentage of mature neutrophils (band and segmented cells) from 5% to 30% while decreasing the percentage of blast-like cells from 85% to 35% (Fig. 7B).

siRNA Knockdown of Cdk1 Induces Terminal Differentiation of Human Myeloid Leukemia YNH-1 Cells with t(16;21) Translocation

We have shown that specific inhibition of Cdk1 activity by a dominant negative Cdk1 and by siRNA could eliminate the differentiation blockage caused by TLS-ERG in mouse L-G myeloid cells. To test whether loss of Cdk1 can also force terminal differentiation of human myeloid leukemia cells with the t(16;21) translocation, we studied the effect of Cdk1 knockdown by siRNA in YNH-1 cells.

YNH-1 is a human myeloid leukemia cell line established from peripheral blood of a leukemia patient carrying a t(16;21) translocation that generates the TLS-ERG fusion protein (15). The expression of the *TLS-ERG* gene in YNH-1 cells was confirmed by reverse transcription-PCR (Fig. 8A) and by Western blotting (Fig. 8B). We generated two lentiviral constructs that coexpress GFP as well as siRNAs that targets human Cdk1. The effectiveness of these siRNAs to downregulate human Cdk1 was confirmed after examination by Western blotting (Fig. 8C). As we have done with L-G myeloid cells, lentivirus-infected YNH-1 cells were enriched by flow cytometry sorting and used for Wright-Giemsa staining. Although YNH-1 cells harboring the scrambled siRNA largely maintained the myeloblast-like morphology, down-regulation of Cdk1 protein by siRNA knockdown resulted in a 2.5-fold increase of segmented neutrophils even in the absence of G-CSF induction (Fig. 8D).

Cell Cycle Arrest Is Required for Restoring Differentiation to TLS-ERG Cells

As a critical cell cycle molecule, down-regulation of Cdk1 should lead to decreased cell proliferation or even cell cycle

arrest. Indeed, we observed that 5-aza-2′-deoxycytidine or trichostatin A was able to down-regulate Cdk1 and stop the growth of L-G cells harboring the empty LXSN retroviral vector or those expressing TLS-ERG (Fig. 5B and C and data not shown). We therefore examined the cell cycle profile before and after treatment with these two epigenetic drugs. In cells harboring the empty LXSN retroviral vector or those expressing TLS-ERG, treatment with 5-aza-2′-deoxycytidine or trichostatin A resulted in a decrease of cells in the S phase and an increase in G_2-M (Fig. 9A), suggesting that these cells were being arrested at the G_2-M transition of the cell cycle.

For TLS-ERG cells infected with a lentivirus for siRNA expression, we noticed that the percentage of GFP^{+} cells harboring a scrambled siRNA had minimal change after several
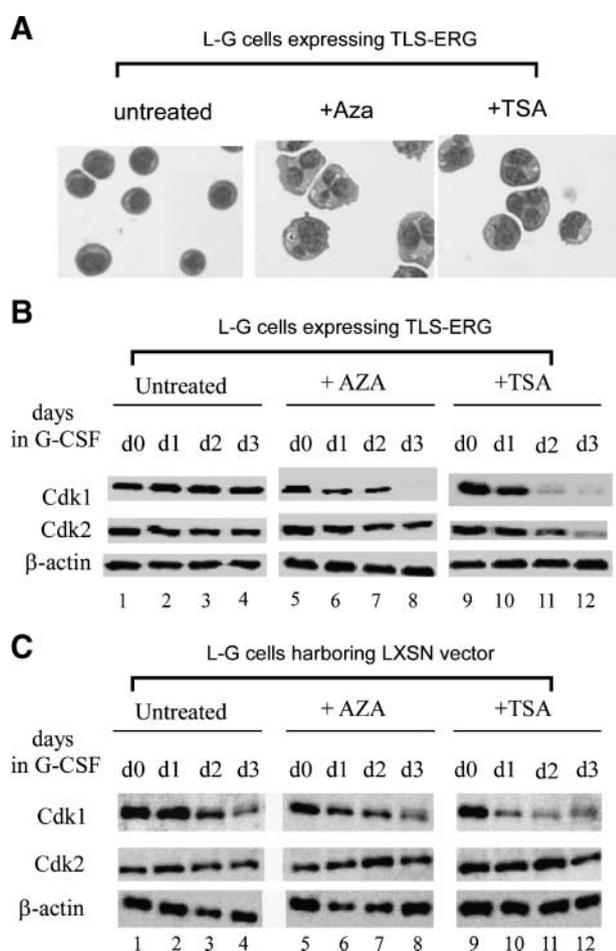
FIGURE 5. Restoration of terminal differentiation with inhibitors of DNA methyltransferase and histone deacetylase. **A.** L-G cells expressing TLS-ERG were cultured in G-CSF for 7 d without any treatment (*left*), or cultured in G-CSF with 5-aza-2′-deoxycytidine (2.5 μmol/L) for 5 d (*middle*), or with trichostatin A (25 ng/mL) for 3 d (*right*) before Wright-Giemsa staining to observe morphology changes. **B.** Whole-cell lysates were prepared from TLS-ERG–expressing L-G cells without any treatment (*lanes 1-4*), treated with 5-aza-2′-deoxycytidine (*lanes 5-8*), or with trichostatin A (*lanes 9-12*) for Western blotting with specific antibodies. **C.** Whole-cell lysates were prepared from control L-G cells harboring the empty LXSN retroviral vector without any treatment (*lanes 1-4*) or from cells treated with 5-aza-2′-deoxycytidine (*lanes 5-8*) or with trichostatin A (*lanes 9-12*) for Western blotting.
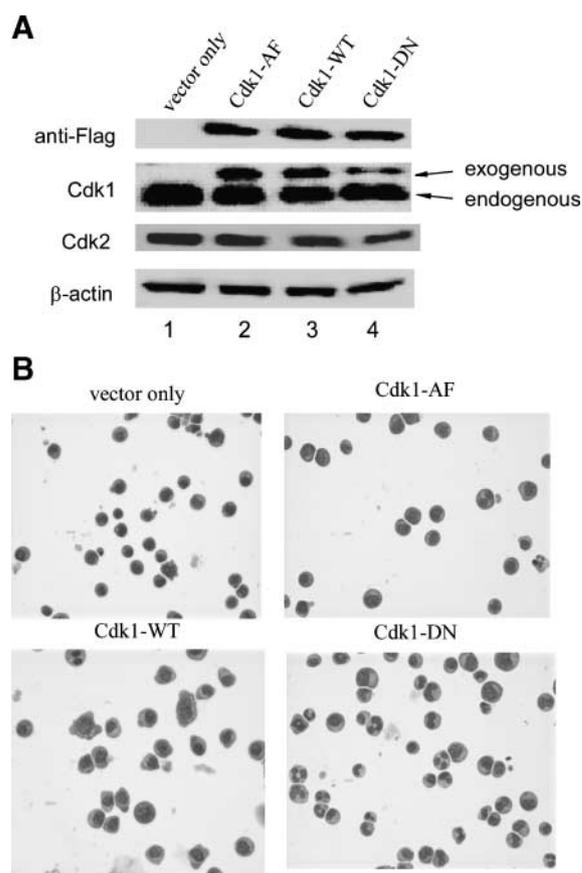
FIGURE 6. Restoration of terminal differentiation by dominant-negative Cdk1 in TLS-ERG–expressing L-G cells. **A.** TLS-ERG cells were infected with an empty lentiviral vector (*lane 1*) or lentivirus harboring Flag-tagged constitutively active Cdk1-AF (*lane 2*), wild-type Cdk1-WT (*lane 3*), or the dominant-negative Cdk1-DN (*lane 4*). After enrichment of GFP^{+} cells through sorting by flow cytometry, whole-cell lysates were prepared for Western blotting with the M2 anti-Flag antibody to detect exogenous Flag-Cdk1 and with anti-Cdk1 antibody for comparison with endogenous Cdk1. **B.** TLS-ERG cells harboring empty lentiviral vector or expressing Cdk1-AF, Cdk1-WT, and Cdk1-DN were collected after sorting and cultured in G-CSF for 3 d before Wright-Giemsa staining to observe morphologic changes.

days of culture. However, the percentage of GFP^{+} cells harboring siRNA against Cdk1 decreased rapidly in culture (from 25% to 5% in 2 days). To examine the differences in cell cycle status between these two cell populations that express different siRNAs, we enriched GFP^{+} cells through fluorescence-activated cell sorting. After analysis of cell cycle, we found that cells harboring the siRNA against Cdk1 had a lower percentage in the G_1 phase and a higher percentage in the G_2-M phase than those harboring the scrambled siRNA (Fig. 9B), suggesting that Cdk1 knockdown negatively affects proliferation and causes some of these cells to be arrested at the G_2-M phase.

Overexpression of dominant-negative Cdk1 by transient transfection has been reported to arrest cells at the G_2-M transition in most cell types (13). In TLS-ERG cells, we noticed that GFP[+] population expressing dominant-negative Cdk1-DN grew slower than GFP+ cells expressing wild-type Cdk1, but we were unable to detect an increase of cells in G_2-M. We suspect that the number of GFP^{+} cells with a sufficiently high

level of Cdk1-DN expression is too small to cause a discernible shift in cell cycle profile.

The above finding prompted us to ask whether any approach to induce cell cycle arrest could restore terminal differentiation to TLS-ERG–expressing L-G cells, or whether TLS-ERG contributes more than continual proliferation. For this, we tested TLS-ERG cells with a membrane-permeable cyclic AMP (cAMP) analogue dibutyryl-cAMP (Bt$_2$cAMP). This cAMP analogue has been reported to cause growth arrest of macrophages in the mid-G$_1$ stage of the cell cycle (16). When tested in TLS-ERG–expressing L-G cells, Bt$_2$cAMP decreased the number of cells in the S phase and arrested cells in the G$_1$ phase (Fig. 10A). Although G-CSF alone was unable to force terminal differentiation of TLS-ERG cells, addition of Bt$_2$cAMP to G-CSF medium induced these cells to undergo terminal differentiation (Fig. 10B). In addition, we also noticed a decrease of Cdk1 in these cells after Bt$_2$cAMP treatment (Fig. 10C). These results suggest that the major function of TLS-ERG is to sustain Cdk1 expression, deregulate the cell cycle, and confer a proliferative advantage. It appears that TLS-ERG–induced changes are not irreversible. As long as the cell cycle is arrested, TLS-ERG cells can be induced to undergo terminal myeloid differentiation.

Discussion

Cdk1 (historically known as cdc2) is a member of the cyclin-dependent Ser/Thr kinase family. Cdk1 was originally identified as a catalytic subunit of the highly conserved protein kinase complex known as the M-phase promoting factor (17, 18). The kinase activity of Cdk1 is controlled at several levels, namely (*a*) at the level of regulatory Cdk1 phosphorylations; (*b*) at the level of activation through binding to cyclins such as B1; and (*c*) at the level of inactivation by inhibitors such as p21^{cip1} and p27^{Kip1}.

The cell division cycle is a fundamental and highly complex process that is conserved in all eukaryotic cells. The conventional view is that in mammalian cells, progression through G$_1$ phase is driven by the activities of Cdk4 and Cdk6, which associate with D-type cyclins. Entry into the S phase and initiation of DNA replication requires the activity of Cdk2, which is activated by E-type cyclins in the late G$_1$ and S phases and by A-type cyclins in the S and G$_2$ phases. Finally, entry into M phase requires the activity of Cdk1, which associates with both A- and B-type cyclins. However, such a view has been challenged by recent findings that mice lacking Cdk2 or Cdk4 are viable and develop with minimal defects (19-22). Cdk1 can functionally substitute for Cdk2 in the Cdk2$^{-/-}$ background (23). On the other hand, Cdk1 appears to be the predominant kinase in cell cycle and is essential for cell survival as mouse embryos lacking Cdk1 died at a very early stage during the development and Cdk1$^{-/-}$ cells are not viable (24, 25). These recent findings suggest that Cdk2 or other Cdks do not have the same compensatory capacity as Cdk1.

The critical role of Cdk1 in terminal myeloid differentiation is further supported by the present study demonstrating that TLS-ERG transformation of L-G cells is accompanied by sustained expression of Cdk1, and down-regulation of Cdk1 by 5-aza-2′-deoxycytidine or trichostatin A leads to restoration of

terminal differentiation. In addition, dominant-negative inhibition of Cdk1 kinase activity and siRNA knock-down of Cdk1 protein both are capable of unlocking differentiation blockage imposed by TLS-ERG. It should be noted that although these treatments all lead to cell cycle arrest at the G$_2$-M phase, our experiment with Bt$_2$cAMP indicate that restoration of terminal differentiation is not G$_2$-M arrest specific and arrest at the G$_1$ phase can also restore G-CSF–induced terminal differentiation to TLS-ERG cells.

Because leukemogenesis involves at least two hits (genetic mutations), isolating the effects of TLS-ERG from other cooperating events may be difficult without knowing the nature of other genetic changes. In this study, we have found that a key function of TLS-ERG is to disrupt control of the cell cycle through deregulation of Cdk1. The availability of our TLS-ERG mouse leukemia model makes it possible to test the effect
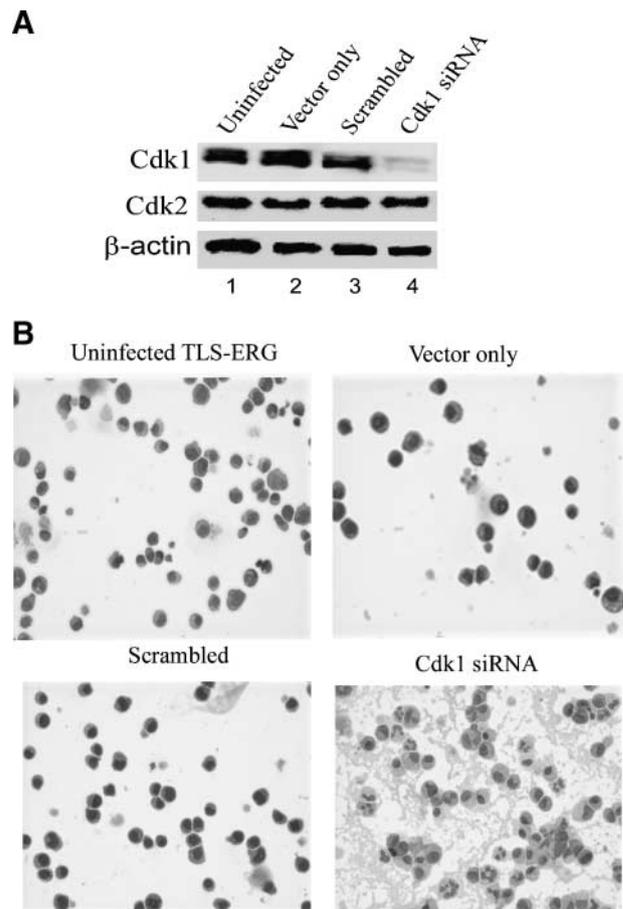
FIGURE 7. Restoration of terminal differentiation by siRNA knockdown in TLS-ERG–expressing L-G cells. **A.** TLS-ERG cells were not infected (*lane 1*) or infected with an empty lentiviral vector (*lane 2*), with lentivirus expressing a scrambled siRNA (*lane 3*), or with a siRNA targeting mouse Cdk1 (*lane 4*). After enrichment of GFP$^+$ cells through sorting by flow cytometry, whole-cell lysates were prepared for Western blotting with anti-Cdk1 to confirm knockdown of Cdk1 protein. **B.** TLS-ERG cells were not infected or infected with an empty lentiviral vector, with lentivirus expressing the scrambled siRNA, or with a siRNA targeting mouse Cdk1. GFP$^+$ cells were collected after sorting and cultured in G-CSF for 3 d before Wright-Giemsa staining to observe morphologic changes. Fold increase of mature neutrophils (band and segmented cells) was calculated after counting of 200 cells.
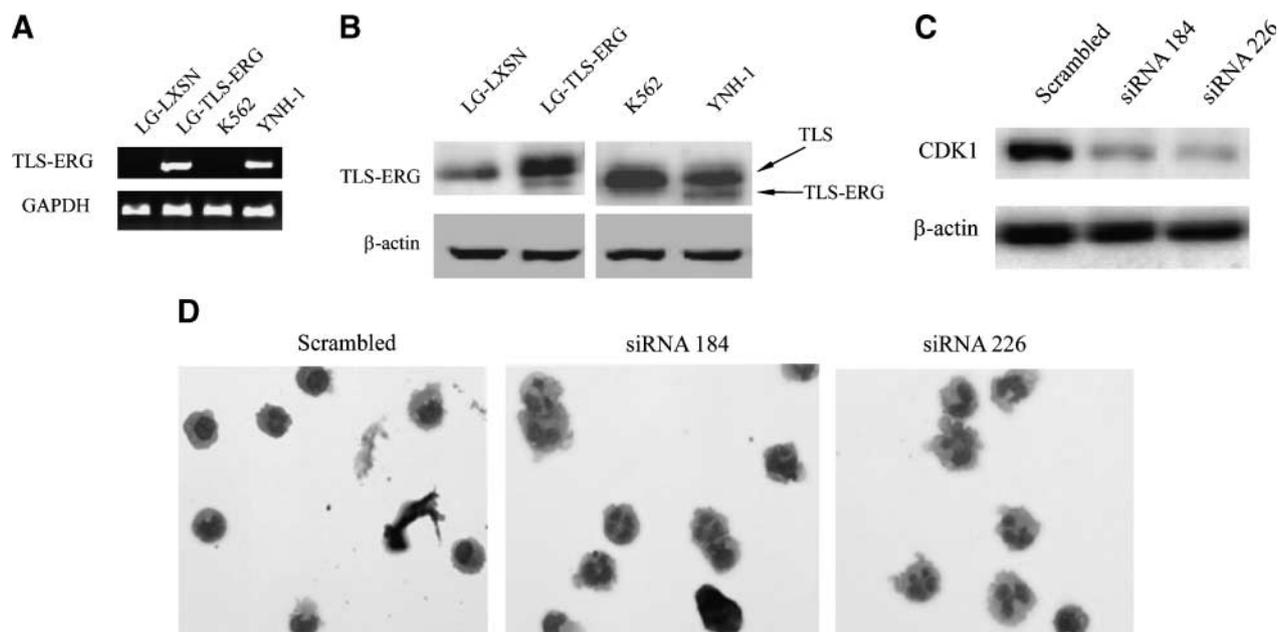
FIGURE 8.   Effects of Cdk1 knockdown in human YNH-1 leukemia cells. **A.** Total RNAs from L-G cells harboring LXSN vector or expressing TLS-ERG (*lanes 1* and *2*), K562 (*lane 3*), and YNH-1 (*lane 4*) cells were isolated. The expression of TLS-ERG mRNA was analyzed by reverse transcription-PCR. **B.** Nuclear extract was prepared from L-G cells harboring LXSN or expressing TLS-ERG (*lanes 1* and *2*) or from human K562 (*lane 3*) and YNH-1 (*lane 4*) cells. The proteins were then separated and blotted with a rabbit polyclonal α-TLS antibody that recognizes both TLS and TLS-ERG. **C.** Lysates from 293 cells expressing a scrambled siRNA (*lane 1*) or siRNAs targeting human Cdk1 (*lanes 2* and *3*) were blotted with an anti-Cdk1 antibody to show knockdown of Cdk1. **D.** YNH-1 cells were infected with lentivirus expressing the scrambled siRNA (*left*) or siRNAs targeting human Cdk1 (*middle* and *right*). After culture for 3 d in IL-3 medium, GFP+ cells were sorted for morphologic examination by Wright-Giemsa staining. Fold increase of segmented neutrophils was calculated after counting 200 cells.

of TLS-ERG on the initiation/progression of a leukemia-like disease through inducible siRNA knockdown of Cdk1 and through treatment with small molecule drugs such as inhibitors of DNA methylase, histone deacetylase and Cdk1 kinase, or with molecules that can bring arrest to the cell cycle. Although further experiments are needed to investigate how TLS-ERG deregulates Cdk1 expression in myeloid cells, our findings from this study should be useful in the search of new treatment strategies for acute myeloid leukemia characterized by the t(16;21) chromosomal translocation and by the resultant TLS-ERG fusion.

Materials and Methods

Cell Culture

Mouse L-G myeloid progenitor cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies), 1% penicillin/streptomycin, 2 mmol/L L-glutamine, and 1 ng/mL recombinant mouse IL-3 (R&D Systems) at 37°C in a humidified atmosphere with 5% CO_2. Human YNH-1 cells were kindly provided by Dr. Hiroyuki Hamaguchi (15) and cultured in RPMI 1640 with 10% fetal bovine serum, 10% horse serum, 1 mmol/L sodium pyruvate, 1% penicillin/streptomycin, 2 mmol/L L-glutamine, and 10 ng/mL recombinant human IL-3. BOSC23 retroviral packaging cells were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% FCS plus 0.025 mg/mL mycophenolic acid (Sigma) and 2.176 μg/mL aminopterin (Sigma). 293FT lentiviral packaging cells were obtained from Invitrogen.

Retrovirus

pLXSN-FL-TLS-ERG retroviral plasmid expressing Flag (FL) epitope-tagged TLS-ERG was constructed by cloning full-length TLS-ERG cDNA into the *Eco*RI site of the LXSN vector. For generation of retrovirus, BOSC23-packaging cells were transfected with pLXSN constructs by the calcium phosphate precipitation method. After 48 h, supernatants containing retroviral particles were collected and used to infect L-G cells in the presence of 6 μg/mL polybrene. Infected cells were selected in medium containing 1 mg/mL G418, and G418-resistant clones were pooled in this study to rule out potential bias caused by clonal variations.

Lentivirus

Lentiviral siRNA was obtained by annealing the predesigned primers and subsequent cloning into the *Hpa*I-*Xho*I sites of the pLL3.7 lentiviral vector (14) that also coexpresses GFP as a selection marker. Primers targeting nucleotides 200 to 218 of mouse Cdk1 mRNA are 5′-TGAAGAAGATCAGACTTGA-ATTCAAGAGATTCAAGTCTGATCTTCTTCTTTTTTC-3′ (sense) and 5′-TCGAGAAAAAAGAAGAAGATCAGACTT-GAATCTCTTGAATTCAAGTCTGATCTTCTTCA-3′ (antisense). Primers targeting nucleotides 184 to 202 of human Cdk1 mRNA are 5′-TACTTCGTCATCCAAATATATTCAAGA-GATATATTTGGATGACGAAGTTTTTTTC-3′ (sense) and 5′-TCGAGAAAAAAACTTCGTCATCCAAATATATCTCTT-GAATATATTTGGATGACGAAGTA-3′ (antisense). Primers targeting nucleotides 226 to 244 of human Cdk1 mRNA are

5'-TGCAGGATTCCAGGTTATATTTCAAGAGAATA-TAACCTGGAATCCTGCTTTTTTTC-3' (sense) and 5'-TCGA-GAAAAAAGCAGGATTCCAGGTTATATTCTCTTGAAATA-TAACCTGGAATCCTGCA-3' (antisense).

For construction of lentivirus expressing Flag-epitope tagged Cdk1, human cDNA inserts encoding wild-type (WT), constitutively active (AF), or dominant-negative (DN) Cdk1 were released from pcDNA3.1 vector (Invitrogen) by digestion with *Eco*RI and *Pst*I, and cloned in-frame into the pSG5-FL vector (6) for addition of the Flag-epitope to the NH$_2$-terminal end. The resultant pSG5-FL-Cdk1 plasmid was then digested with *Sal*I and cloned into the *Xho*I site of a modified pLL3.7 lentiviral vector from which the U6 siRNA promoter was removed by *Xba*I-*Hpa*I digestion. For the generation of lentivirus, 293FT packaging cells were transfected with 3 μg of pLL3.7 construct plus 9 μg ViraPower DNA Mixture (Invitrogen) by the Lipofectamine 2000 method according to the manufacturer's instructions. After 48 h, supernatants containing lentiviral particles were collected and used to infect L-G cells in the presence of 10 μg/mL polybrene. Infected GFP$^+$ cells were enriched through sorting by flow cytometry.

## Analysis of Cell Growth and Morphologic Changes

The growth of L-G cells harboring the empty retroviral vector or expressing TLS-ERG was monitored daily under different experimental conditions, and viable cells were identified using the trypan blue dye exclusion method. For morphologic changes after different treatments, 2×10^4 cells were collected onto a glass slide using a cytofunnel and automatically processed for Wright-Giemsa staining on a Hematek Stain Pak machine. Cell morphology was examined under Olympus BX51TF microscope and the images were acquired using Basic SPOT software.

In vivo Leukemogenesis Assay

Eight-week-old BALB/c mice (eight males and eight females) purchased from Harlan Sprague were syngeneic with L-G cells and were used for the *in vivo* leukemogenesis experiments. Cells (1×10^6) were resuspended in 0.1 mL PBS and inoculated by tail vein injection. Moribund animals were euthanized; tissue and organs were first weighed and then collected for histologic staining. The animal use protocols had been reviewed and approved by the Institutional Animal Care and Use Committee.
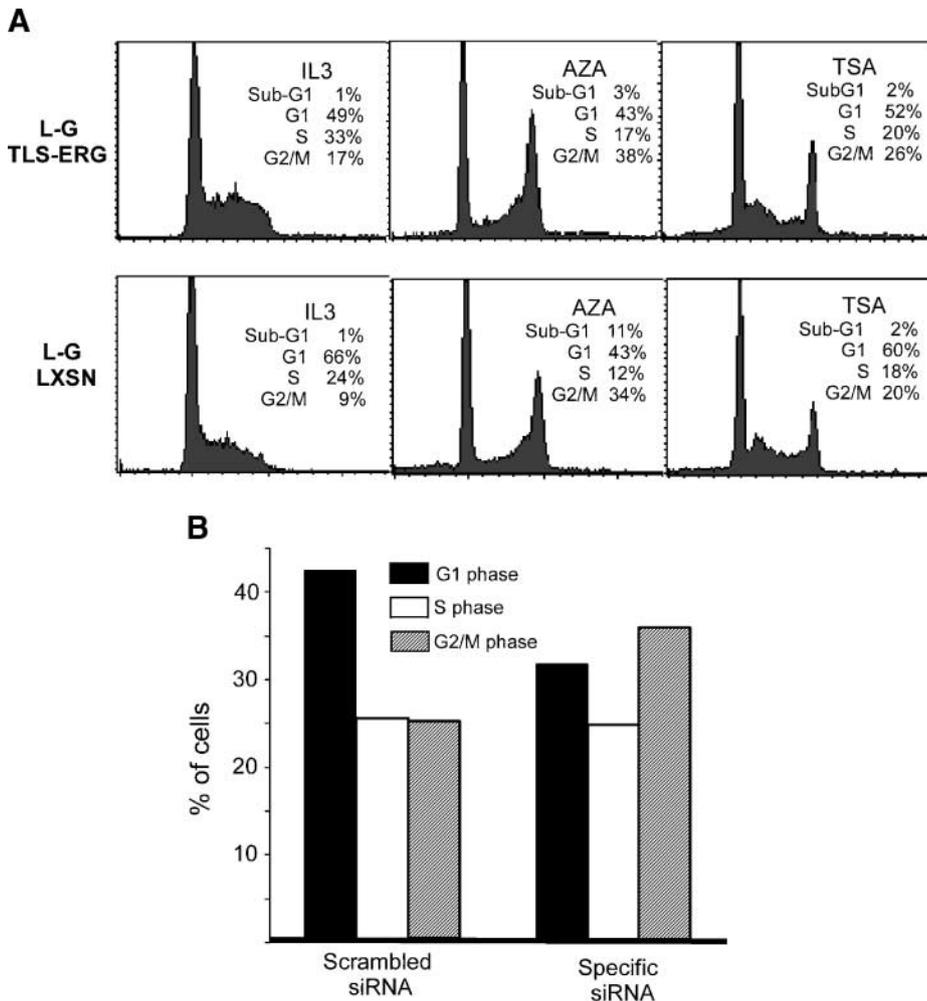
FIGURE 9. Cell cycle profiles of TLS-ERG−expressing cells after different treatments. **A.** TLS-ERG−expressing L-G cells grown in IL-3 medium were washed thrice with PBS, then placed in medium containing G-CSF (10 ng/mL) and 2.5 μmol/L 5-aza-2'-deoxycytidine (*AZA*) or 25 ng/mL trichostatin A (*TSA*). After 16 h, cells were collected for cell cycle analysis (*top*). L-G cells harboring an empty LXSN retroviral vector were similarly treated to show that AZA and TSA have similar effects on the cell cycle in these control cells. **B.** TLS-ERG−expressing L-G cells were infected with lentivirus expressing a scrambled siRNA or a siRNA targeting Cdk1. Following 2 d of culture in IL-3 medium after the infection, cells were washed and replaced in G-CSF medium for 24 h, and GFP$^+$ cells were enriched through sorting and used in cell cycle analysis. Results of cell profile are from a typical experiment.
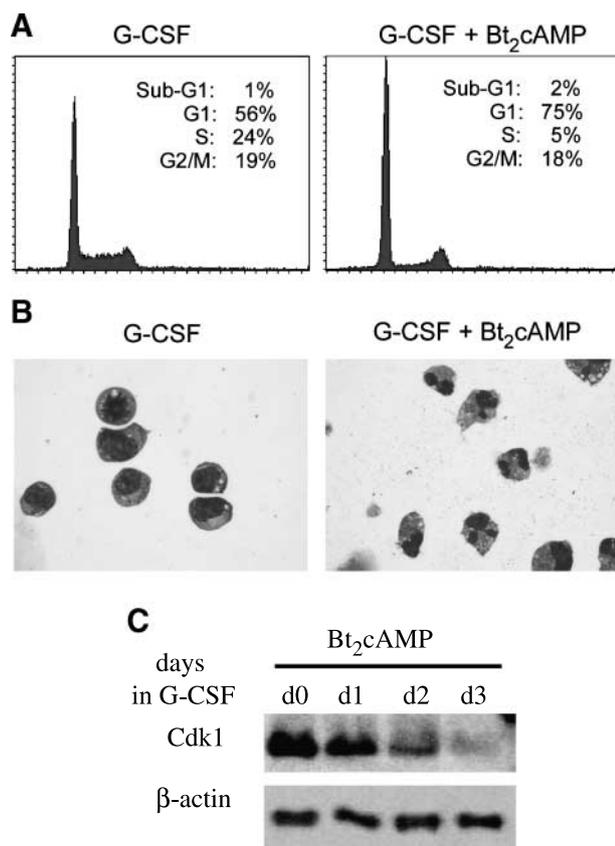
FIGURE 10. Cell cycle arrest and restoration of terminal differentiation by Bt2cAMP in TLS-ERG–expressing cells. **A.** TLS-ERG–expressing L-G cells grown in IL-3 medium were washed thrice with PBS, then placed in medium containing G-CSF (10 ng/mL) in the presence or absence of 1 mmol/L Bt$_2$cAMP. After 24 h, cells were collected for analysis of cell cycle status. **B.** Cells were cultured in G-CSF medium with or without Bt$_2$cAMP for 4 d, and then subjected to Wright-Giemsa staining for examination of morphologic changes. **C.** Cells were treated with 1 mmol/L Bt$_2$cAMP for the indicated time in G-CSF (10 ng/mL), then lysed for Western blotting with anti-Cdk1.

Reverse Transcription-PCR

Total RNAs were extracted from 2.5×10^6 L-G or YNH-1 cells using the RNeasy Mini Kit (Qiagen) and eluted in 40 μL H$_2$O. For reverse transcription-PCR, 1.5 μL RNA were analyzed in a total volume of 25 μL using the SuperScript One-Step reverse transcription-PCR kit (Invitrogen). Reverse transcription was carried out at 50°C for 30 min and the reverse transcription product was denatured at 94°C for 2 min. PCR amplification of DNA was done at 94°C, 30 s; 55°C, 30 s; and 72°C, 30 s. Primers used in this study include TLS, 5′-GGTGGCTATGAACCCAGAGG-3′ (sense); ERG, 5′-GCGCCACAAAGTTCATCTTCT-3′(antisense); and GAPDH, 5′-GTGGATATTGTTGCCATCATT-3′ (sense) and 5′-TGATGGCAACAATATCCACTT-3′(antisense).

Flow Cytometry

For cell cycle analysis, L-G cells were collected after different treatments, washed with cold PBS twice, and fixed overnight in 70% cold ethanol at −20°C. Fixed cells were then treated in PBS supplemented with 1% RNase A and stained

with 50 μg/mL propidium iodide (Sigma) at 37°C for 30 min. Cell cycle distribution was determined on a FACSCalibur (Becton Dickinson).

Antibodies and Western Blotting

M2 mouse monoclonal anti-Flag antibody was from Sigma (1:1,000 dilution); rabbit anti-TLS antibody (1:1,000 dilution) against the NH$_2$-terminal domain of TLS was obtained from Dr. Moreau-Gachelin (Inserm U830-Institut Curie, Paris, France; ref. 26). All other antibodies in this study were from Santa Cruz Biotechnology, Inc., and include mouse monoclonal anti-Cdk1 (clone 17, 1: 200 dilution), anti-Cdk2 (D-12, 1:200 dilution), and anti–β-actin (AC15, 1:1,000 dilution). For nuclear extract, 2×10^7 cells were collected and lysed in 5 mL NP40 lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40]. The resultant nuclear pellet was resuspended in 0.1 mL buffer X [50 mmol/L Tris (pH 7.4), 270 mmol/L NaCl, and 0.5% Triton X-100] supplemented with protease inhibitors and phosphatase inhibitors. For whole-cell lysates, 1×10^7 cells were lysed with 0.2 mL buffer X. Ten micrograms of each sample were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After blotting with the antibody, protein bands were visualized using the ECL Western Blotting Analysis System from Amersham Pharmacia Biotech.

In vitro *Kinase Assay*

Histone H1 phosphorylation, as a measure for Cdk1 activity, was assayed using a kit from Upstate Biotechnology following the manufacturer's instructions. Briefly, equal amount of lysates (500 μg) from L-G-LXSN and L-G-TLS-ERG cells were incubated with 1 μg of mouse anti-Cdk1 antibody (clone 17) and 40 μL of protein A/G beads at 4°C overnight. The immunoprecipitates were washed twice with lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40], once with kinase buffer [50 mmol/L Tris-HCl (pH 7.6), 10 mmol/L MgCl$_2$, and 5 mmol/L DTT], and resuspended in 20 μL assay dilution buffer. Ten microliters of the immunoprecipitates were then incubated with 10 μL substrate, 10 μL phosphatase inhibitors, and 10 μL of [^{32}P]ATP mixture (75 mmol/L magnesium chloride, 500 μmol/L [^{32}P]ATP) at 30°C for 30 min. Twenty-five microliters of the reaction were transferred onto the center of a P81 phosphocellulose paper and washed thrice with 0.75% phosphoric acid and once with acetone for 5 min. Radioactivity retained on the phosphocellulose paper was measured in a liquid scintillation counter (LS 6000 SC, Beckman Coulter).

Disclosure of Potential Conflicts of Interest

Acknowledgments

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