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Phosphoinositide-dependent kinase 1 regulates leukemia stem cell maintenance in MLL-AF9-induced murine acute myeloid leukemia

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ABSTRACT

Although great efforts have been made to improve available therapies, the mortality rate of acute myeloid leukemia (AML) remains high due to poor treatment response and frequent relapse after chemotherapy. Leukemia stem cells (LSCs) are thought to account for this poor prognosis and relapse. Phosphoinositide-dependent kinase 1 (PDK1) is a critical regulator of the PI3K/Akt pathway and has been shown to be frequently activated in leukemia. However, the role of PDK1 in the regulation of LSCs in AML is still not clear. Using a *PDK1* conditional deletion MLL-AF9 murine AML model, we revealed that the deletion of *PDK1* prolonged the survival of AML mice by inducing LSC apoptosis. This was accompanied by the increased expression of the pro-apoptotic genes *Bax* and *p53* and the reduced expression of *Stat5*, which has been shown to be constitutively activated in leukemia. Thus, our findings suggest that PDK1 plays an essential role in maintaining LSCs. Further delineating the function of PDK1 in LSCs may provide a new strategy for the improved treatment of AML relapse.

1. Introduction

Acute myeloid leukemia (AML) is the most common myeloid disorder, and it is characterized by a clonal disorder of the hematopoietic cells. In AML, hematopoietic progenitors are unable to differentiate into normal mature cells and cause severe bone-marrow failure [1]. The conventional treatment for AML patients is chemotherapy, which is cytotoxic and intolerable. However, many patients still die due to disease relapse. Therefore, there is an urgent need for new therapeutic strategies aiming to eradicate AML. Like the normal hematopoietic system, leukemia stem cells (LSCs) have self-renewal ability, allowing for leukemia cell propagation in AML. LSCs are thought to account for the poor prognosis and relapse of AML because they are insensitive to therapeutic agents [2]. Therefore, understanding the regulation of LSCs will provide useful information for improving the prognosis and preventing relapse in AML patients.

In leukemia, dysregulated pathways that promote proliferation and survival are often linked with the induction of leukemia [3]. Among these pathways, the PI3K/Akt pathway is frequently activated during leukemia development. Under the stimulation of growth factors and cytokines, phosphatidylinositol 3-kinase (PI3K) is activated and recruits Akt to the plasma membrane. Akt is then phosphorylated at its S473 and T308 residues by mTORC2 and Phosphoinositide-dependent kinase 1 (PDK1), respectively [4,5]. After activation, Akt can regulate multiple biological processes through its downstream effectors [6]. Several inhibitors targeting the PI3K/Akt pathway are reported to have therapeutic potential for AML treatment, including Akt inhibitors [7]. PTEN is a negative regulator of the PI3K/Akt pathway. PTEN inactivation is frequently detected in AML and other hematopoietic neoplasms [8], while murine bone marrow cells containing the PTEN mutation can also cause leukemia-like diseases [9]. SHIP, another negative regulator of the Akt pathway, is also frequently inactivated in AML [10]. Akt activation is detected in 50% of AML samples and is associated with a poor prognosis [11]. The activation of Akt in primary AML patients is linked with the dysregulation of other signaling cascades, including p53, NF-κB and BAD [12,13].

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PDK1 is responsible for the phosphorylation of AGC family kinases, including SGK, S6K, PKC and Akt. PDK1 regulates multiple biological processes, including cell survival and proliferation, through multiple downstream substrates. It has been reported that PDK1 is involved in multiple cancers, including breast cancer [14] and prostate cancer [15]. Reducing the expression of *PDK1* could successfully prolong the survival rate and prevent tumor development in PTEN$^{+/-}$ mice [16], indicating that PDK1 is a promising anticancer target. PDK1 is overexpressed in 40% of AML patients associated with poor prognoses, as the survival of AML cells is helped by PDK1 overexpression [17]. These findings strongly suggest that PDK1 might be a potential target for AML therapy. However, the role of PDK1 in the development and maintenance of AML still needs to be addressed. In this study, we examined the role of PDK1 in AML development using a MLL-AF9 murine AML model in which PDK1 is conditionally deleted by pIpC treatment. We found that PDK1 promoted AML development by maintaining the LSCs.

2. Materials and methods

2.1. Mice

PDK1$^{flox/flox}$ C57BL/6J mice [18] were kindly provided by Dr. Dario R. Alessi. *Mx1-Cre* mice [19] were purchased from the Jackson Laboratory. *PDK1*$^{flox/flox}$ mice were crossed with *Mx1-Cre* mice to generate the *Mx1-Cre;PDK1*$^{flox/flox}$ mice and littermate *PDK1*$^{flox/flox}$ mice (the control mice). The detection of the *Mx1* gene was performed by PCR using primers GGTCGATGCAACGAGTGATG and CCAGAGACGGAAATCCATCG. The detection of the *PDK1* gene was performed by PCR using primers TGTGCTTGGTGGATATTGAT and AAGGAGGAGAGGAGGAATGT. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Hematology, Chinese Academy of Medical Science and all mice were hosted in the SPF facilities in the same institute.

2.2. The MLL-AF9 AML mouse model

MLL-AF9 retroviruses were generated in 293T cells transfected with the MSCV-MLL-AF9-IRES-GFP plasmid using Lipofectamine 2000 (Invitrogen). Retroviruses were used to infect bone marrow lineage-negative cells from *PDK1*$^{flox/flox}$ and *Mx1-Cre;PDK1*$^{flox/flox}$ mice. MLL-AF9-infected cells were injected intravenously into lethally irradiated (9.6 Gy) C57BL/6J recipients. The primary MLL-AF9 AML cells were isolated from the BM and spleen of recipients and injected into secondary sub-lethally irradiated recipients. Seven days after the transplantation, secondary recipients were intraperitoneally (i.p.) injected with polyinosinic-polycytidylic acid (pIpC, Amersham) four times every other day to delete the *PDK1* gene.

2.3. Flow cytometry

Single cell suspensions from the blood, spleen or bone marrow were isolated, washed and stained with fluorochrome-labeled antibodies according to the expression of surface or intracellular markers in PBS supplemented with 2% fetal bovine serum. All flow cytometric experiments were performed on a FACS-Canto II, LSR II or LSR Fortessa for analysis and FACS-Aria III for sorting (BD Biosciences). The data were analyzed using Flowjo software.

2.4. Cell cycle and apoptosis assay

GFP$^+$ BM cells from AML recipients were stained with Gr-1 and c-Kit antibodies to identify LSCs. After staining, the cells were stained with DAPI and Ki67 to determine the cell-cycle profile. For the apoptosis assay, GFP$^+$ BM cells labeled with Gr-1 and c-Kit antibodies were washed and stained with Annexin V and 7-AAD at room temperature followed by flow cytometric analysis.

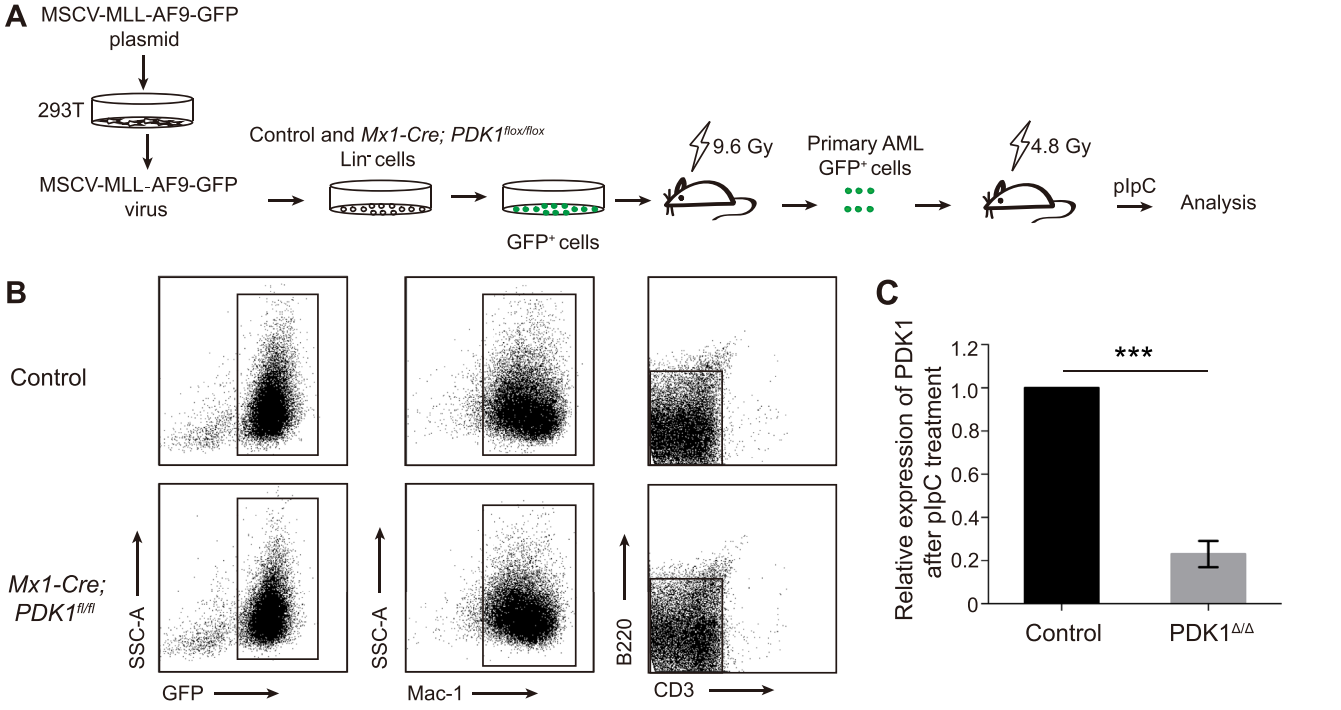

Fig. 1. Establishment of the *Mx1-Cre;PDK1*$^{flox/flox}$-MLL-AF9 AML mouse model. (A) BM cells from *PDK1*$^{flox/flox}$ and *Mx1-Cre;PDK1*$^{flox/flox}$ mice were transformed with MSCV-MLL-AF9-GFP retroviruses. Sorted GFP$^+$ cells were then transplanted into lethally irradiated mice by tail vein injection. After AML development, GFP$^+$ cells from the spleen of AML primary recipients were sorted and transplanted into sub-lethally irradiated mice, and pIpC were treated 1 week after transplantation. (B) GFP$^+$ cells from BM of primary leukemia mice were stained with Mac-1, B220 and CD3 for flow cytometric analysis ($n = 5$). (C) Real-time RT-PCR was performed to examine the efficiency of the *PDK1* excision in GFP$^+$ AML cells 7 days after pIpC treatment ($n = 5$). Data are shown as the mean ± SD (***$P < 0.001$).

2.5. Real-time RT-PCR

Real-time RT-PCR was performed using SYBR GREEN PCR Master Mix (Roche) to examine the mRNA expression levels. Changes between the groups of relative gene expression were calculated using $2^{-\Delta\Delta CT}$ methods normalized based on the *gapdh* expression.

2.6. Statistical analysis

Statistically significant differences in the parameters measured between the groups were assessed using an unpaired Student's test. Significance was denoted with asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Establishment of the Mx1-Cre;PDK1$^{flox/flox}$-MLL-AF9 AML mouse model

To examine the role of PDK1 in AML development, we first crossed *PDK1*$^{flox/flox}$ mice with Cre transgenic mice driven by the *Mx1* promoter (an inducible promoter which is activated upon pIpC treatment). To establish the conditional *PDK1* knockout AML model, lineage-negative BM cells from *Mx1-Cre;PDK1*$^{flox/flox}$ mice and littermate *PDK1*$^{flox/flox}$ mice (the Controls) were transduced with MLL-AF9-GFP retroviruses. GFP^{+} MLL-AF9 cells were sorted and transplanted into lethally irradiated recipients (Fig. 1A). After transplantation, both recipient mice developed AML and succumbed within 30 days. Flow cytometric analysis revealed that the GFP^{+} cells from the recipient mice were Mac-1^{+}, CD3^{-} and B220^{-}, indicating the development of AML in the recipient mice (Fig. 1B). *Mx1-Cre;PDK1*$^{flox/flox}$-MLL-AF9 mice were treated with pIpC to delete the *PDK1* gene (indicated as the PDK1$^{\Delta/\Delta}$ mice). Real-time RT-PCR analysis demonstrated the efficient deletion of the *PDK1* gene in sorted GFP^{+} cells from *Mx1-Cre;PDK1*$^{flox/flox}$-MLL-AF9 mice (Fig. 1C).

To exclude the effect of *Mx1-Cre* expression on AML development, we analyzed the percentage of survival, total leukemia cells (GFP^{+}) and LSCs in BM of mice that had been transplanted with *PDK1*$^{flox/flox}$-MLL-AF9 cells or *Mx1-Cre*-MLL-AF9 cells. No difference was found in these two groups, indicating that *Mx1* expression by pIpC treatment has no effect on mouse survival and leukemia development (Supplemental Fig. 1A–C). To test whether Mx1-Cre was activated without pIpC treatment, we also examined the expression of *PDK1* in *Mx1-Cre;PDK1*$^{flox/flox}$-MLL-AF9 cells without pIpC treatment and found that the expression level of PDK1 was comparable to that of the controls (Supplemental Fig. 2A). In addition, there were no differences in the survival rate, GFP^{+}

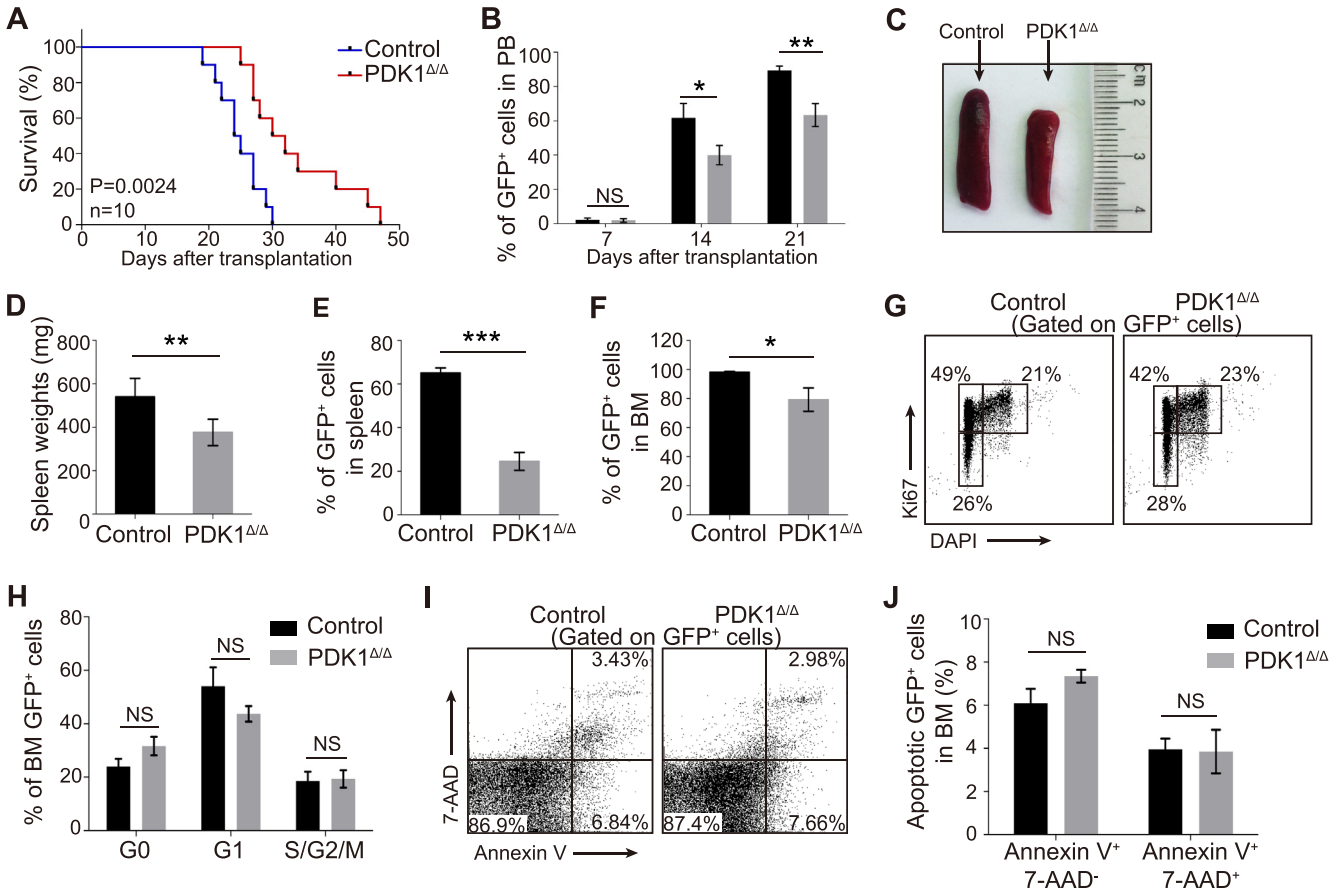

Fig. 2. PDK1 is required for the MLL-AF9 development *in vivo*. (A) Kaplan–Meier survival curves for recipients injected with control or *Mx1-Cre;PDK1*$^{flox/flox}$ leukemia cells treated with pIpC (thus becoming PDK1$^{\Delta/\Delta}$) starting on day 7 of the leukemia cell injection ($n = 10$). (B) The percentage of GFP^{+} cells in PB at the indicated time point after injection of leukemia cells ($n = 5$). (C) Representative image of the spleens from the control mice (left) and PDK$^{\Delta/\Delta}$-MLL-AF9 leukemia mice (right) 21 days after the leukemia cell injections. (D) Histogram showing the weights of the spleens from the control and PDK$^{\Delta/\Delta}$-MLL-AF9 leukemia mice 21 days after the leukemia cell injections ($n = 5$). (E–F) Histograms showing the frequency of GFP^{+} cells in the spleen and BM of PDK$^{\Delta/\Delta}$-MLL-AF9 and control leukemia mice 21 days after the leukemia cell injections ($n = 5$). (G–H) Representative FACS plots and histograms showing the cell cycle status of GFP^{+} cell in BM of PDK$^{\Delta/\Delta}$-MLL-AF9 and control leukemia mice ($n = 5$). (I–J) Representative FACS plots and histograms showing the cell apoptosis status of GFP^{+} cell in BM of PDK$^{\Delta/\Delta}$-MLL-AF9 and control leukemia mice ($n = 5$). All data are shown as the mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant).

percentage or LSC proportion between the control mice and *Mx1-Cre;PDK1$^{flox/flox}$*-MLL-AF9 mice without pIpC treatment (Supplemental Fig. 2B–D), confirming that there was no spontaneous *Mx1* expression in our experimental groups.

3.2. Deletion of PDK1 inhibits the proliferation of AML cells in vivo

To determine whether PDK1 is involved in the maintenance of MLL-AF9 AML, we transplanted 1 × 10^6 *Mx1-Cre;PDK1$^{flox/flox}$*-MLL-AF9 or control cells into sub-lethally irradiated recipients. Mice recipients were treated with pIpC to excise the *PDK1* gene 7 days after transplantation. Control mice transplanted with MLL-AF9 AML cells developed AML and succumbed within 30 days after transplantation. In contrast, the *PDK1* deletion significantly prolonged the survival of MLL-AF9 AML mice (Fig. 2A) and decreased the percentage of GFP$^+$ cells in PB after the pIpC treatment (Fig. 2B). In addition, the *PDK1*-deficient AML recipients exhibited less splenomegaly than the recipients of the control AML cells (Fig. 2C, D). FACS analysis revealed a significantly lower percentage of GFP$^+$ cells in the BM and spleen of PDK1$^{\Delta/\Delta}$-MLL-AF9 mice than that in the controls (Fig. 2E, F). However, we did not observe any significant changes in the cell cycle status (Fig. 2G, H) or cell apoptosis rates of GFP$^+$ cells from PDK1$^{\Delta/\Delta}$-MLL-AF9 mice in comparison with the control (Fig. 2I, J).

3.3. PDK1 deletion results in a decrease in the LSCs

MLL-AF9 leukemia was found to be maintained by LSCs enriched in the c-Kit$^+$Gr-1$^-$ (K$^+$G$^-$) cell fraction [20]. To explore the effect of *PDK1* deficiency on LSCs, we examined the percentage of LSCs in the BM and spleen of the PDK1$^{\Delta/\Delta}$-MLL-AF9 and control mice. We found that the *PDK1* deletion resulted in a significant decrease in the BM LSCs (Fig. 3A, B) but a lesser decrease in the spleen LSCs (Fig. 3C, D). To further analyze the role of *PDK1* in LSCs, we transplanted 1 × 10^4 c-Kit$^+$Gr-1$^-$ cells from PDK1$^{\Delta/\Delta}$-MLL-AF9 and control primary recipients into secondary sub-lethally irradiated recipients. Consistent with the primary transplantation results, the *PDK*1 deletion also significantly prolonged the survival of the PDK1$^{\Delta/\Delta}$-MLL-AF9 recipients (Fig. 3E) when compared to the controls. Accordingly, the percentage of LSCs in secondary PDK1$^{\Delta/\Delta}$-MLL-AF9 recipients was significantly reduced when compared to the controls (Fig. 3F). These results indicate that PDK1 is involved in the maintenance of LSCs in MLL-AF9 leukemia.

3.4. Deletion of PDK1 induces apoptosis in LSCs

To explore the mechanisms by which PDK1 affects the maintenance of LSCs in MLL-AF9 leukemia, we first analyzed the cell-cycle status in *PDK1*-deficient LSCs by Ki67 and DAPI staining. The percentage of cells in the G0 stage, G1 stage and S/G2/M stage were

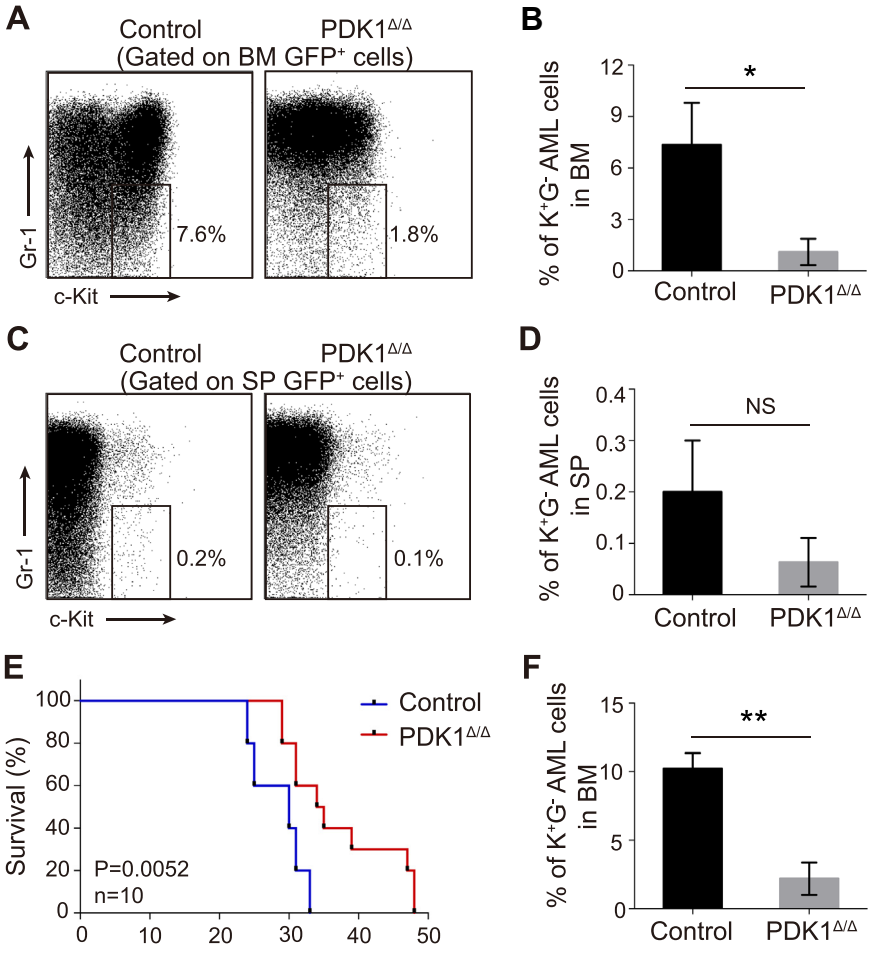

Fig. 3. *PDK1* deletion suppresses LSCs in MLL-AF9 leukemia. (A–B) Representative FACS plots and histograms showing the percentage of LSCs (c-Kit$^+$Gr-1$^-$) in the BM of PDK$^{\Delta/\Delta}$-MLL-AF9 and control leukemia mice ($n = 5$). (C–D) Representative FACS plots and histograms showing the percentage of LSCs (c-Kit$^+$Gr-1$^-$) in the spleen of PDK$^{\Delta/\Delta}$-MLL-AF9 and control leukemia mice ($n = 5$). (E) Kaplan–Meier survival curves for recipients injected with 1 × 10^4 PDK$^{\Delta/\Delta}$ or control LSCs ($n = 10$). (F) Histograms showing the frequency of LSCs (c-Kit$^+$Gr-1$^-$) in the BM of mice injected with PDK$^{\Delta/\Delta}$ or control LSCs ($n = 5$). All data here are shown as the mean ± SD (*$P < 0.05$; **$P < 0.01$; NS, not significant).

similar between the PDK1$^{\Delta/\Delta}$-MLL-AF9 LSCs and controls (Fig. 4A, B), indicating that the *PDK1* deletion did not affect the cell-cycle progression of LSCs. We next examined the apoptosis of LSCs without *PDK1*. We found significantly increased levels of early apoptosis (Annexin V$^+$7-AAD$^-$) and late apoptosis (Annexin V$^+$7-AAD$^+$) in the *PDK1*-deficient LSCs compared with the control cells (Fig. 4C, D), suggesting that *PDK1* deletion impairs LSC maintenance by specifically inducing apoptosis.

To further probe how the loss of PDK1 affects the apoptosis pathway in LSCs, we sorted LSCs from PDK1$^{\Delta/\Delta}$-MLL-AF9 mice and control mice and analyzed the expression profiles of known pro-survival genes and pro-apoptotic genes. Compared with the controls, the *PDK1* deletion increased the expression of the pro-apoptotic genes *Bax* and *p53* in LSCs (Fig. 4E), suggesting that PDK1 prevents apoptosis by suppressing *Bax* and *p53* expression in LSCs. In addition, we examined the expression level of several transcription factors involved in the regulation of hematopoietic cells. We found that the expression level of *Stat5* was significantly lower in the *PDK1*$^{\Delta/\Delta}$ LSCs when compared with that of the control LSCs (Fig. 4F). These results indicate that PDK1 might prevent apoptosis of LSCs in MLL-AF9 leukemia by suppressing the expression of *Bax* and *p53* and activating *Stat5*.

4. Discussion

In AML, it is known that the constitutive activation of the PI3K/Akt pathway enhances the proliferation and survival of leukemia cells [21]. While PDK1 is essential for the maximum activity of Akt by phosphorylating its T308 residue, the role of PDK1 in the regulation of AML remains unclear. By conditionally deleting *PDK1* in MLL-AF9 murine leukemia model, we found that *PDK1*-deficient leukemia mice had a longer lifespan compare to the controls, with significant decreases in the leukemia cell loads of both the BM and spleen. Secondary transplantation experiments clearly demonstrated that *PDK1* deletion caused a reduction of the LSC frequencies in AML. These findings revealed that PDK1 is required for the development of AML by maintaining LSCs.

LSCs have been demonstrated to be responsible for maintaining the propagation of leukemia, although they only account for a minor proportion of AML cells [22]. Multiple pathways have been shown to be involved in the regulation of LSCs, including the Wnt pathway, the NF-κB pathway and the Adherens junction pathway [23]. The dysregulation of these pathways in LSCs may fuel their ability for self-renewal and proliferation [24]. Emerging evidence has supported the idea that leukemia relapse is mediated by the surviving LSCs, a common phenomenon after the chemical leukemia therapy. The drug-resistant LSCs eventually outgrow the leukemia cells and become dominant during relapse [25]. Because most LSCs are quiescent, insensitive to chemotherapy and divide infrequently, they are difficult to eradicate. It is thus essential to find a treatment that targets LSCs. In this study, we found that the deletion of *PDK1* could significantly decrease the proportion of LSCs in BM. As a consequence, the lifespan of leukemia mice was prolonged with decreased leukemia cells. Furthermore, we showed that the deletion of *PDK1* significantly up-regulated the expression of *Bax* and *p53*, known mediators of apoptosis induction [26]. *p53*

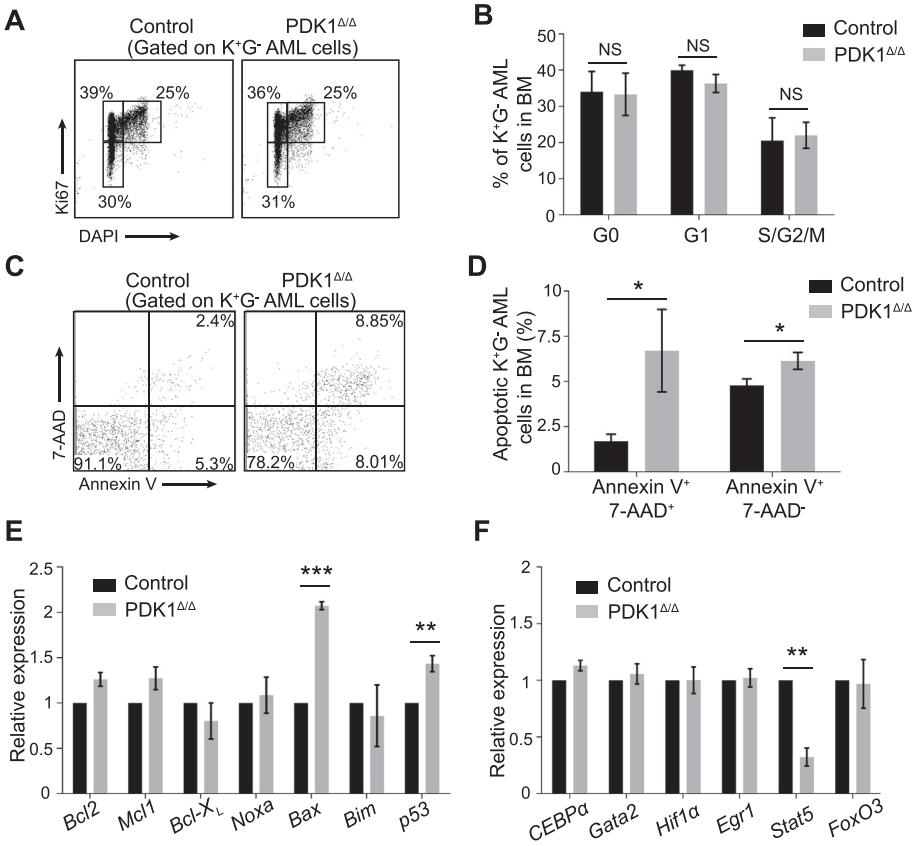

Fig. 4. PDK1 is required for the maintenance of LSCs in MLL-AF9 leukemia. (A–B) Representative FACS plots and histograms showing the cell cycle status of BM LSCs of PDK$^{\Delta/\Delta}$-MLL-AF9 and control leukemia mice ($n = 5$). (C–D) Representative FACS plots and histograms showing the cell apoptosis status of BM LSCs of PDK$^{\Delta/\Delta}$-MLL-AF9 and control leukemia mice ($n = 5$). (E–F) Real-time RT-PCR was performed to detect the expression of apoptosis-related genes and transcription factors in sorted LSCs (c-Kit$^+$Gr-1$^-$). All data here are shown as the mean ± SD (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant).

elevation in tumors has been shown to be a good response indicator for cancer chemotherapies in comparison with *p53*-deficient tumors [27], while the low expression of *Bax* is coincident with a poor drug response and shortened survival [28]. Thus, our findings highlight the role of PDK1 in the apoptosis signaling pathway in MLL-AF9 AML and indicate that PDK1 may serve as a promising drug target for preventing relapse and prolonging survival in leukemia patients, as PDK1 mainly regulates leukemia development by maintaining the LSCs.

In this study, we observed decreased *Stat5* expression after *PDK1* deletion in LSC cells. This raised the possibility that PDK1 might maintain LSCs through Stat5. STAT family proteins, also family members of the Jak/STAT pathway, regulate cell differentiation, growth and survival in multiple cell types. Stat5 has frequently been seen to be constitutively activated in lymphoid and myeloid leukemia of both acute and chronic types [29] and promotes oncogenesis [30]. Akt has been shown to be required for the activation of Stat5 [31], while the Stat5-Akt signaling cascade is essential for cell growth and survival [32]. In addition, Stat5 also has anti-apoptotic or pro-survival roles in the apoptosis process [33,34]. Thus, we speculate that PDK1 may regulate LSC survival in a Stat5-dependent manner through Akt. Further studies are needed to support this hypothesis. Taken together, we have demonstrated a critical role of PDK1 in the maintenance of LSCs in AML via regulating LSC apoptosis. Our study supports the idea that relapsed AML patients might benefit from PDK1-specific inhibitors.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.007.

Transparency document

The Transparency Document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.03.007.

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