ARTICLE

CD244 maintains the proliferation ability of leukemia initiating cells through SHP-2/p27^{kip1} signaling

Feifei Zhang,[1*] Xiaoye Liu,[1*] Chiqi Chen,[1] Jun Zhu,[2] Zhuo Yu,[1] Jingjing Xie,[3] Li Xie,[1] Haitao Bai,[2] Yaping Zhang,[1] Xia Fang,[4] Hao Gu,[1] Chun Wang,[2] Wei Weng,[1] Cheng Cheng Zhang,[5] Guo-Qiang Chen,[1] Aibing Liang[4] and Junke Zheng[1]

[1]Hongqiao International Institute of Medicine, Shanghai Tongren Hospital, Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Faculty of Basic Medicine, Shanghai Jiao Tong University School of Medicine, China; [2]Department of Hematology, The 1st People's Hospital, Shanghai Jiao Tong University School of Medicine, China; [3]Binzhou Medical University, Taishan Scholar Immunology Program, Yantai, China; [4]Department of Hematology, Tongji Hospital of Tongji University School of Medicine, Shanghai, China and [5]Departments of Physiology and Developmental Biology, UT Southwestern Medical Center, Dallas, TX, USA

FZ and XL contributed equally to this work

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ABSTRACT

Targeting leukemia initiating cells is considered to be an effective way to cure leukemia, for which it is critical to identify novel therapeutic targets. Herein, we demonstrate that CD244, which was initially reported as a key regulator for natural killer cells, is highly expressed on both mouse and human leukemia initiating cells. Upon CD244 knockdown, human leukemia cell lines and primary leukemia cells have markedly impaired proliferation abilities both *in vitro* and *in vivo*. Interestingly, the repopulation ability of both mouse and human hematopoietic stem cells is not impaired upon CD244 knockdown. Using an MLL-AF9-induced murine acute myeloid leukemia model, we show that leukemogenesis is dramatically delayed upon CD244 deletion, together with remarkably reduced Mac1$^+$/c-Kit$^+$ leukemia cells (enriched for leukemia initiating cells). Mechanistically, we reveal that CD244 is associated with c-Kit and p27 except for SHP-2 as previously reported. CD244 co-operates with c-Kit to activate SHP-2 signaling to dephosphorylate p27 and maintain its stability to promote leukemia development. Collectively, we provide intriguing evidence that the surface immune molecule CD244 plays an important role in the maintenance of stemness of leukemia initiating cells, but not in hematopoietic stem cells. CD244 may represent a novel therapeutic target for the treatment of acute myeloid leukemia.

Introduction

It was suggested that leukemia initiating cells (LICs) or leukemia stem cells (LSCs) are responsible for initiation, development and relapse of leukemia. Identification of novel theraputic targets specific to LICs is the key for the eradication of leukemia. We previously identified that an immune inhibitory receptor, LILRB2, played an essential role in the stemness maintenance of LICs and hematopoietic stem cells (HSCs).[1] Studies from other groups also suggest that targeting certain surface immune molecules of LICs may be an attractive way to block leukemogenesis.[2,3] However, an ideal therapeutic target for leukemia treatment should only be essential for maintaining the pool of LICs, but not for HSCs. We hypothesized that other surface immune molecules, including both inhibitory and stimulatory recep-

Correspondence:
chengq@shsmu.edu.cn/
zhengjunke@shsmu.edu.cn/
lab7182@tongji.edu.cn

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tors, may function differentially between HSCs and LICs.[4] It is urgent to understand such molecules for the eradication of LICs.

CD244 is one of the immune molecules initially identified on natural killer (NK) cells, a subset of CD8 T cells and monocytes. It has been reported that CD244 is also highly expressed on human HSCs, but may not (or may rarely) be expressed on mouse HSCs,[5-7] although its function is still not clear. CD244 is a member of the CD2 subset of the immunoglobulin superfamily with four immune-receptor based tyrosine switch motifs (ITSM)[8] which can function as an activating or inhibitory receptor by alternative adaptor recruitments including SAP, EAT-2, SHP-2 and SHP-1. Human CD244 interacts with SAP to activate NK cells.[9,10] However, in the absence of SAP, CD244 associates with the inhibitory phosphatase SHP-2 or SHP-1 to function as an inhibitory receptor.[11,12] CD244 is also known to associate with PI3K and SHIP1 as this receptor also signals through the inositol phospholipid signaling pathway.[13-15] Nevertheless, the functions of CD244 in leukemia development and the potential targets involved in CD244/SHP-2 signaling remain largely unknown.

In this study, we showed that knockdown of CD244 by shRNAs induced a marked decrease of the proliferation abilities in both human AML cell lines and primary LICs, but not in mouse and human HSCs. Furthermore, CD244 deletion remarkably delayed leukemogenesis and depleted LICs in a murine MLL-AF9-transduced AML model. CD244 co-operates with c-Kit and initiates downstream SHP-2/p27 signaling to manipulate the activities of LICs. Therefore, CD244 may be an ideal target for the eradication of acute myeloid LICs.

Methods

Lentivirus construction, infection and *in vivo* xenograft

The lentiviral vector PLL3.7 was used to express shRNAs designed to target CD244 (sequences are listed in *Online Supplementary Table S1*). Using a calcium phosphate transfection method, lentivirus constructs together with the packaging plasmids of pSPAX2 and pMD2G were mixed and transfected into 293T cells. Lentiviruses were used for the following infection on human leukemia cell lines, human cord blood CD34$^+$ HSCs or acute myeloid leukemia (AML) samples. Human cord blood CD34$^+$ HSCs were purified by using CD34 enrichment Kit (Miltenyi). Details of the clinical sample information are listed in *Online Supplementary Table S2*. Total AML cells from Samples #12 to #16, #19 and #20 (subtype, M5) were used for infection, *in vitro* colony assay and *in vivo* transplantation. MV4-11 cells ($2.5x10^6$), human cord blood CD34$^+$ HSCs ($2x10^5$) or primary AML cells ($2x10^6$) were resuspended in 200 μL or 50 μL (for primary AML cells) PBS and transplanted into sublethally irradiated (250 rad) NOD-SCID mice by either retro-orbital or intra-tibial injection. All the mice were sacrificed for determination of engraftment at 2-3 months post transplantation.

Retroviral infection, transplantation and flow cytometric analysis

MLL-AF9-expressing retroviruses were produced in 293T cells with an MSCV-MLL-AF9-IRES-YFP encoding plasmid.[16] Lin$^-$ fetal liver cells were isolated from wild-type (WT) and CD244 knockout (KO) mice and infected with MLL-AF9 retroviruses by two rounds of spinoculation in the presence of 4 μg/mL polybrene. Infected cells (200,000) were transplanted into lethally irradiated (1000 rad) C57BL/6 mice by retro-orbital injection. Indicated YFP$^+$ bone marrow cells from primary transplanted mice were further infused into recipient mice for secondary transplantation or limiting dilution analysis. Flow cytometry and cell cycle analyses were performed as we described previously.[1] For analysis of lineages and LICs, either peripheral blood or bone marrow cells were stained with anti-mouse Mac-1-APC, anti-mouse Gr-1-PE, anti-mouse CD3-APC, anti-mouse B220-PE, or anti-mouse c-Kit-PE antibodies (eBioscience). CD244 expression on human LICs or HSCs were labeled by the antibodies against human CD45 (FITC), Lineage marker (APC), CD34 (eFluor 450), CD38 (PE-Cy7), CD90 (PE-Cy5.5), CD45RA (PE), CD123 (PE) and CD244 (Biotin). Expression of CD244 and c-Kit on mouse or human LICs (or cell lines) were detected by anti-mouse CD244-PE or human CD244-APC and anti-human c-Kit-PE antibodies (eBioscience). Cell cycle status was measured with Ki-67/7-AAD (or Hoechst 33342) staining (BD Pharmingen) according to the manufacturer's instructions.

Study approval

Bone marrow mononuclear cells were obtained from the patients following diagnostic work at the Department of Hematology at Xinhua Hospital or the 1st People's Hospital; human cord blood were obtained from the Department of Gynaecology and Obstetrics at the 6th People's Hospital, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from all of the patients and all the procedures were approved by the Ethics Committee for Medical Research (IRB) at Shanghai Jiao Tong University School of Medicine.

Methods related to mouse information, western blotting and co-immunoprecipitation, quantitative RT-PCR, colony forming unit assays, bone marrow transplantation and statistical analyses are available in the *Online Supplementary Appendix*.

Results

CD244 is required for the proliferation of both human leukemia cell lines and acute myeloid LICs

To identify novel surface immune molecules (SIMs) that regulate the stemness of HSCs or LICs, we screened approximately 30 potential candidates that were known to be expressed on immune cells.[17] As shown in *Online Supplementary Figure S1A* (mean fluorescence intensity) and Figure 1A (frequencies of SIM$^+$ cells), we found that several immune receptors, including IREM-1, CD244 and JAM3, were highly expressed on both human CD34$^+$ hematopoietic stem/progenitor cells (HSPCs) and AML cells transduced with MLL-AF9 oncogene in human CD34$^+$ HSCs, MA9 cells.[18] Intriguingly, one of these molecules, CD244, which is critical for functions of NK cells, was expressed at the highest level on both HSPCs and AML cells. To elucidate the roles of CD244 in human AML, we first examined the protein levels of CD244 on different human AML cell lines. Most of the AML cell lines expressed CD244, including Kasumi-1 (M2), NB4 (M3), HL-60 (M3), THP-1 (M5), U937 (M5), MV4-11 (M5) and HEL (M6) (Figure 1B). We then constructed several shRNAs to specifically knockdown CD244 to evaluate its roles in cell proliferation. shCD244#1 and shCD244#2 efficiently reduced the CD244 levels as compared with that in scrambled cells, respectively, measured by either flow cytometric analysis or quantitative RT-PCR (Figure 1C and D). Since shCD244#2 had the highest knockdown efficiency, it was mainly used for the following experiments to unravel the functions of CD244 in human leukemia cells.

We then down-regulated the expression of CD244 in MV4-11 cells with shCD244#2 and found that these cells had notable decreased proliferation ability *in vitro* even two days upon CD244 knockdown (Figure 1E). Meanwhile, shCD244#1 had a similar effect when it was used to knock down CD244 in MV4-11 cells (*Online Supplementary Figure S1B*). We also observed similar effects in other CD244-knockdown leukemia cell lines including HL-60, U937 and THP-1 *(Online Supplementary Figure S1C-E)*. We then injected CD244-knockdown MV4-11 cells into NOD-SCID mice and demonstrated that loss of CD244 led to a significantly decreased engraftment compared to that in the control (6.29±4.35% *vs.* 63.90±1.21%) (Figure 1F).

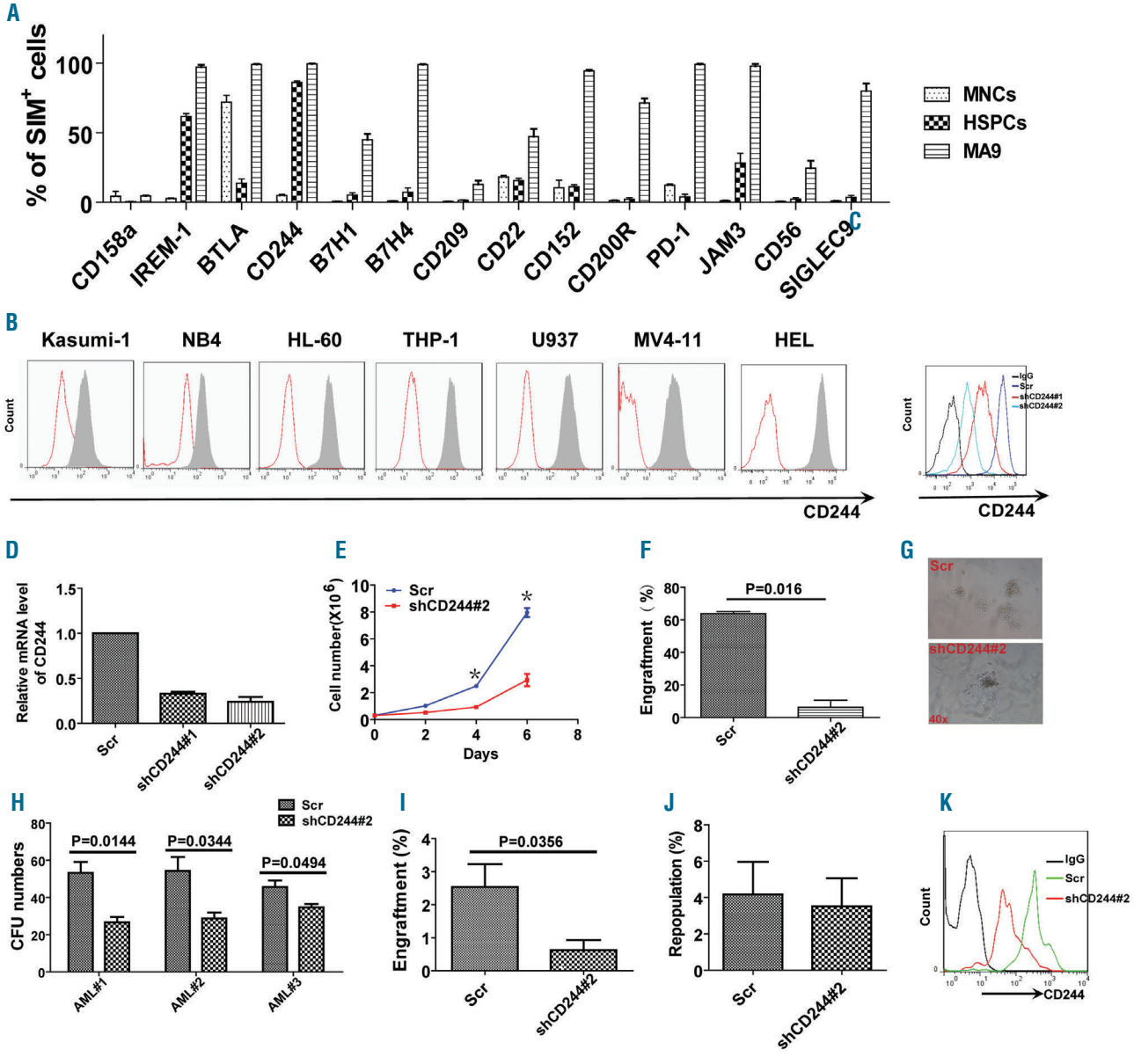

Figure 1. CD244 is required for the proliferation of both human leukemia cell lines and acute myeloid leukemia initiating cells (LICs). (A) Frequencies of representative surface immune molecules on human cord blood hematopoietic stem/progenitor cells (HSPCs) and MLL-AF9-transduced human MA9 cells were measured by flow cytometric analysis. Cord blood mononuclear cells (MNCs) were used for serving as the control of total cell population. (B) Representative flow cytometric analysis of CD244 expression on different leukemia cell lines including Kasumi-1 (M2), NB4 (M3), HL-60 (M3), THP-1 (M5), U937 (M5), MV4-11 (M5) and HEL (M6) (isotype control, red line). (C and D) Knockdown efficiency of CD244 targeted by scrambled shRNA (Scr), shCD244#1 and shCD244#2 was evaluated by either flow cytometric analysis (C) or quantitative real-time RT-PCR (qRT-PCR) as in (D). (E) Cell numbers were counted at indicated days after infection with CD244-targeting shCD244#2 or scrambled shRNA in a representative experiment (n=3). (F) MV4-11 cells infected with CD244-targeting shCD244#2 and control cells were injected into NOD-SCID mice. Three months later, engrafted leukemia cells in the bone marrow were detected by anti-human CD45 antibodies (n=6-8). (G and H) Representative images or colony numbers of patients' CD34$^+$ AML cells were examined after infection with CD244-targeting shCD244#2 or scrambled shRNA in a representative experiment (n=3). (I) Human primary leukemia cells infected with CD244-targeting shCD244#2 and scrambled shRNA were injected into NOD-SCID mice. Two months later, engrafted leukemia cells in the bone marrow were evaluated by flow cytometeric analysis in a representative experiment. (J) Repopulation was evaluated in human cord blood CD34$^+$ cells after infection with CD244-targeting shCD244#2 or scrambled shRNA 12 weeks after transplantation (n=5). (K) Knockdown efficiency of CD244 targeted by scrambled shRNA (Scr) and shCD244#2 was evaluated in the stably engrafted GFP$^+$ CB cells by flow cytometric analysis 12 weeks after transplantation (*P<0.05).

To address the function of CD244 in human AML CD34+ cells, we analyzed the levels of CD244 in different types of human AML samples (M2, M3, M4 and M5) (*Online Supplementary Table S2*) by flow cytometry and found that most of the AML CD34+ cells (enriched for LICs) were positive for CD244. In contrast, most of the tested B-cell acute lymphoblastic leukemia (B-cell ALL) CD34+ cells expressed at a much lower level of CD244 (*Online Supplementary Figure S2A and B*). Interestingly, CD244 also expressed on some CD34- cells including blasts. Because it has been reported that leukemia blasts have differentially expressed surface markers compared to the residual HPSCs, such as CD123 or CD45RA,[19-21] we further examined the CD244 level on the immunophenotypic CD45^{dim}SSC^{low} Lin$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^+$ or Lin$^-$CD34$^+$CD38$^-$CD90$^-$CD123$^+$ CD34$^+$ blasts (CD34$^+$ LICs) and CD45^{dim}SSC^{low} Lin$^-$CD34$^-$CD38$^-$CD90$^-$CD45RA$^+$ or CD45^{dim}SSC^{low} Lin$^-$CD34$^-$CD38$^-$CD90$^-$CD123$^+$ CD34$^-$

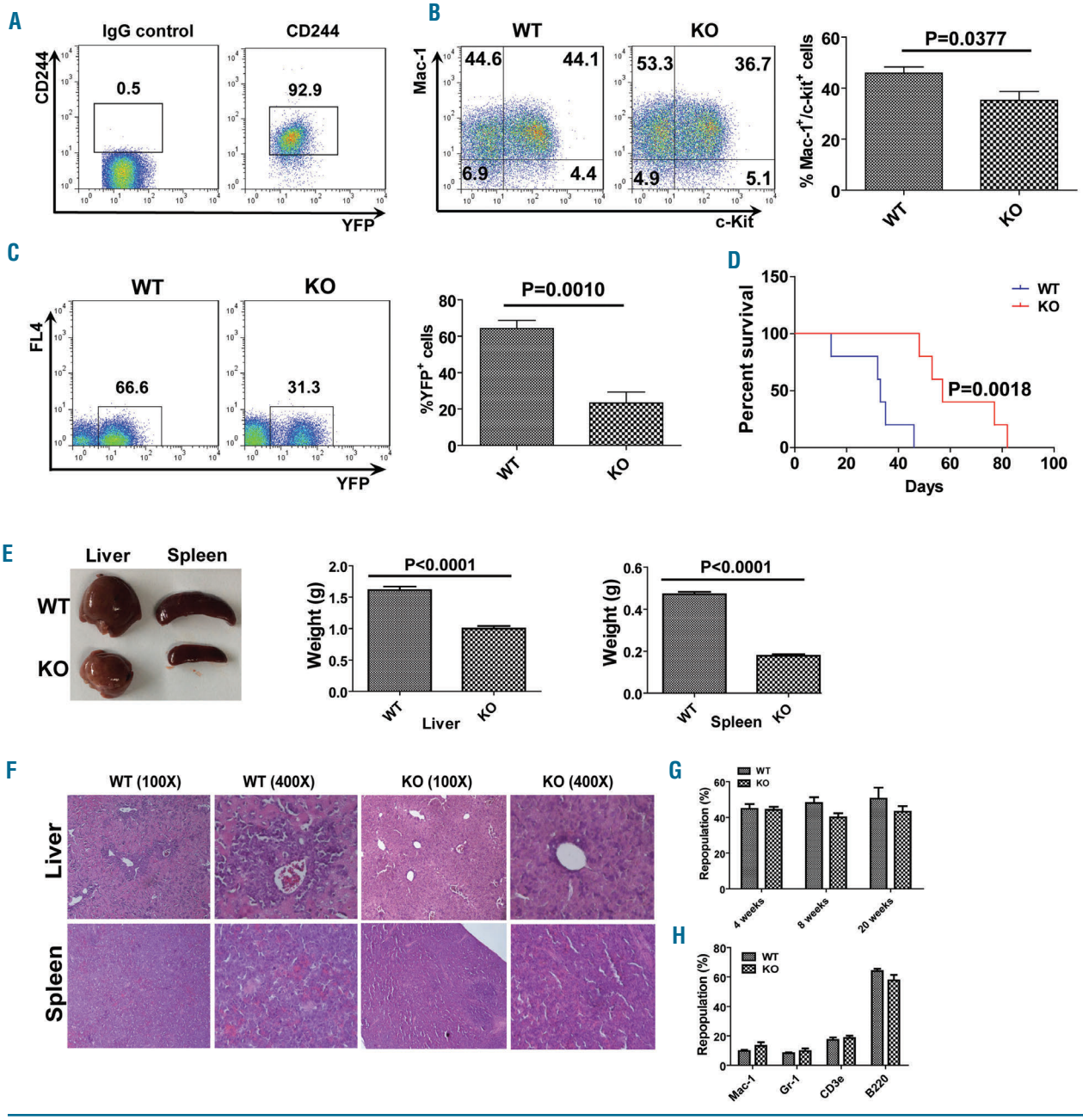

Figure 2. CD244 promotes leukemogenesis in a murine acute myeloid leukemia (AML) model. (A) Representative flow cytometric analysis of CD244 expression in mouse MLL-AF9-transduced wild-type (WT) leukemia cells compared to isotype control. (B) Representative flow cytometric analysis for Mac-1+/c-Kit+ LICs in the bone marrow of primary recipient mice (left). Quantification of the percentage of Mac-1+/c-Kit+ cells from different mice (n=5, right). (C) Representative flow cytometric analysis for detection of YFP+ leukemia cells in peripheral blood of the recipients receiving WT or CD244-null leukemia cells from primary transplantation (left). Quantification of the percentage of leukemia cells (n=5, right). (D) Secondary transplantation of 100 leukemia cells displayed significantly delayed onset of leukemogenesis by CD244-null cells compared to WT cells (n=5, log-rank test). (E) Comparison of the sizes of spleens and livers of the mice transplanted with WT or CD244-null leukemia cells upon secondary transplantation (n=5-7). (F) Histological hematoxylin & eosin staining of AML infiltration in the livers and spleens of mice, as shown in (E). (G) Repopulation was evaluated with WT and CD244-null donor bone marrow cells at indicated time points post transplantation (n=5). (H) Multi-lineages were analyzed in WT and CD244-null donor cells 20 weeks after transplantation (n=5).

blasts (CD34$^-$ LICs). The flow cytometric analysis showed that almost all the CD34$^+$ blasts expressed CD244, while CD34$^-$ blasts had a significantly lower level of CD244 compared to that of CD34$^+$ ones (*Online Supplementary Figure S2C*). Interestingly, CD34$^+$ blasts, but not CD34$^-$ blasts, had similar CD244 expression level with Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ or Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD123$^-$ residual HSCs (*Online Supplementary Figure S2D-F*).

We then knocked down the CD244 levels in several human AML samples with shCD244#1 or shCD244#2. Consistently, the deletion of CD244 led to a notable delay in the growth of CD34$^+$ LICs by shCD244#2 or shCD244#1 (*Online Supplementary Figure S3A*). Moreover, the *in vitro* functional colony forming assay revealed a significant declination in colony numbers (Figure 1G and H). We further performed serial re-plating experiments with human AML cells upon the knockdown of CD244 (*Online Supplementary Figure S3B and C*), which showed that the colony numbers were significantly reduced after serial re-plating (*Online Supplementary Figure S3D*). Importantly, when human AML cells were knocked down by shCD244#2 targeting CD244 and transplanted into NOD-SCID mice, the engraftment was dramatically decreased compared to controls (Figure 1I and *Online Supplementary Figure S3E*). Since CD244 is also expressed on Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^+$ immunophenotypic human HSCs (*Online Supplementary Figure S3F*), but may not be expressed or may be expressed only in some fractions of mouse HSCs,[5-7] we decided to investigate its role in human HSCs as well. Interestingly, no obvious proliferation defects were observed in CD244-knockdown human cord blood HSCs (*Online Supplementary Figure S3G*). Then CD244-knockdown HSCs were infused into NOD-SCID mice and the repopulation was analyzed at 12 weeks post transplantation, showing that CD244 was not required for the repopulation ability of HSCs (Figure 1J and *Online Supplementary Figure S3H and I*). We also examined the knockdown efficiency of CD244 in human HSCs after transplantation, where the stably engrafted GFP$^+$ CB cells infected either by shCD244#2 or Scramble shRNA were gated for the analysis of CD244 expression, which showed that CD244 was decreased to around 67.5% of that in the control (Figure 1K). This may also indicate that the lack of phenotype might not be due to an escape mechanism by CD244-expressing HSCs.

CD244 promotes leukemogenesis in a murine AML model

We further used a transplantable murine AML model driven by the MLL-AF9 oncogene[22] to extensively explore the function of CD244 in leukemia development. We first evaluated the protein levels of CD244 on WT leukemia cells (tagged with YFP) and found that more than 92.9% of them were positive for CD244 as compared to isotype control by flow cytometry (Figure 2A). We further monitored leukemia progression by determining WT and CD244-null (hereafter referred to as KO) circulating leukemia cells in peripheral blood (*Online Supplementary Figure S4A*), which only expressed myeloid cell markers (Mac-1 and Gr-1), but not lymphoid cell markers (CD3 and B220) (*Online Supplementary Figure S4B and C*). Surprisingly, we did not find significant differences either

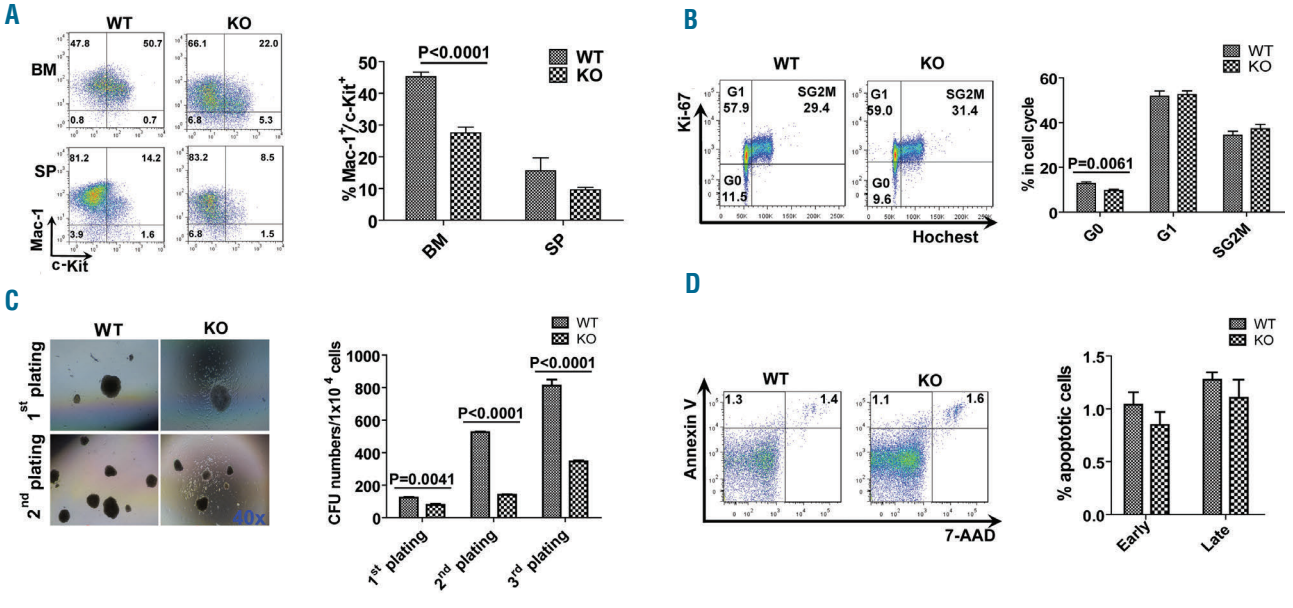

Figure 3. CD244 regulates the proliferation ability of leukemia initiating cells (LICs). (A) Representative flow cytometric analysis for the distribution of wild-type (WT) or CD244-null Mac-1$^+$/c-Kit$^+$ cells (enriched for LICs) in the bone marrow (BM) and spleens (SP) of the recipients upon secondary transplantation (left). Quantification of the percentage of Mac-1$^+$/c-Kit$^+$ cells (n=5-7 for BM and n=3-4 for SP, respectively, right). (B) Representative images for colony forming units for WT and CD244-null acute myeloid leukemia (AML) cells in 1st and 2nd plating (left). Colony numbers were compared in WT and CD244-null AML cells of the secondary recipients after serial plating (n=3, right). (C) Stages of cell cycle were determined by using Ki-67 and Hoechst 33342 staining in WT and CD244-null LICs of the recipients upon secondary transplantation (left). Quantitative analysis of the cell cycle in a representative experiment (n=4, right). (D) Representative flow cytometric analysis for apoptosis of WT or CD244-null Mac-1$^+$/c-Kit$^+$ cells in the bone marrow of the recipients upon secondary transplantation (left). Quantification of the percentages of apoptotic Mac-1$^+$/c-Kit$^+$ cells (n=3, right).

for the frequencies of YFP$^+$ leukemia cells or the differentiation status as compared by the percentages of differentiated Mac-1$^+$/Gr-1$^+$ leukemia cells (*Online Supplementary Figure S4*). There was no significant change in survival between WT and CD244-null leukemic mice as well (*Online Supplementary Figure S4D*). Nevertheless, we observed a notable decrease of Mac-1$^+$/c-Kit$^+$ LICs in the bone marrow of CD244-null primary recipients than WT ones (35.23±3.52% *vs.* 45.88±2.45%) (Figure 2B).

Thereby, we performed a secondary transplantation to further reveal the effect of CD244 in LICs. We found that CD244-null YFP$^+$ leukemia cells in peripheral blood were dramatically reduced compared to WT counterparts at three weeks post transplantation (23.21±5.98% *vs.* 64.26±4.42%) (Figure 2C). Most importantly, recipients of MLL-AF9-transduced CD244-null cells had a markedly extended survival (57 *vs.* 33) (Figure 2D and *Online Supplementary Figure S4E*). We further performed the limiting dilution assay to evaluate the LIC frequency in CD244-null leukemia cells, which was markedly decreased compared to WT controls (1 in 724 *vs.* 1 in 59) (*Online Supplementary Table S3*). Consistently, we also found that the sizes of livers and spleens were much smaller in the mice transplanted with CD244-null AML cells (Figure 2E). This was further confirmed by histological hematoxylin & eosin staining, showing much less infiltration in the mice injected with CD244-null leukemia cells (Figure 2F). These results also indicate the loss of self-renewal ability in CD244-null LICs. Consistent with our observations in human HSCs, CD244-null mouse HSCs also had normal repopulation ability 20 weeks after transplantation (Figure 2G and H). Because we did not uncover drastic changes in primary recipients, we then mainly focused on the phenotypes in secondary recipients.

CD244 promotes the proliferation of LICs

To further characterize the CD244-null AML, we first examined the phenotypic LIC frequencies in the bone marrow and spleens of mice receiving leukemia cells from primary recipients, which was significantly reduced to 60.9% of that in WT counterparts in the bone marrow (27.54±1.60% *vs.* 45.21±1.47%) (Figure 3A). We also

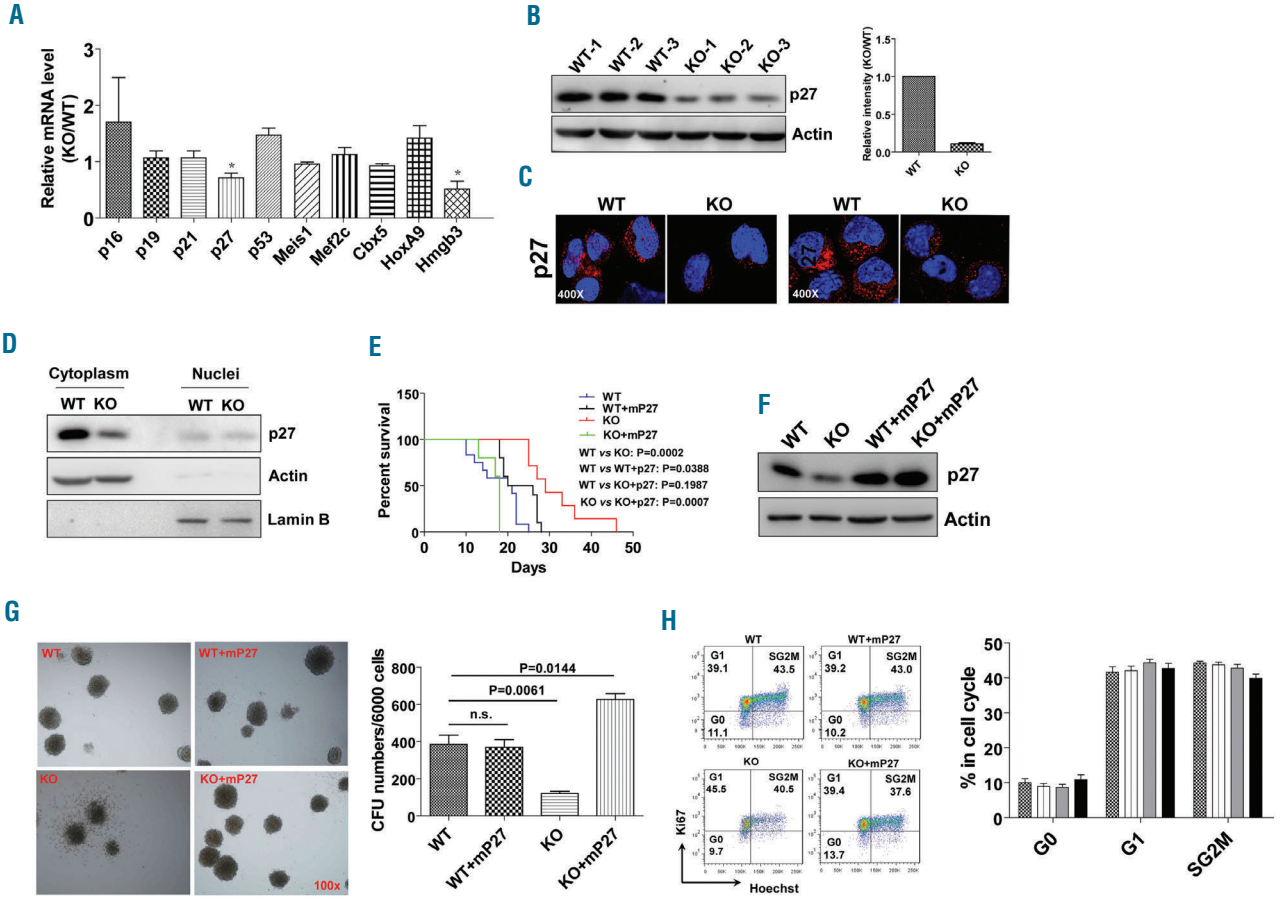

Figure 4. CD244 manipulates p27 levels to maintain the proliferation ability of leukemia-initiating cells (LICs). (A) Potential candidates in wild-type (WT) and CD244-null LICs examined by qRT-PCR. (B) p27 levels were compared between WT and CD244-null LICs by western blotting analysis (left). Quantification of p27 levels between WT and CD244-null LICs (right). (C) p27 levels were compared between WT and CD244-null LICs by immunofluorescence staining in different sets of experiments. (D) Cytoplasmic and nuclear form of p27 of WT and CD244-null LICs were determined by western blotting. (E and F) p27 was over-expressed in both WT and CD244-null AML cells and transplanted into the recipient mice. Survival (E) and p27 levels (F) were analyzed among the mice receiving WT, CD244-null, p27-over-expressed WT and CD244-null AML cells (n=5-10, log-rank test). (G) Colony forming assay were performed *in vitro* and representative images for colonies (left) and colony forming unit (CFU) numbers (right) derived from WT, CD244-null, p27-over-expressed WT and CD244-null AML cells were calculated (n=3). (H) Stages of cell cycle were determined by using Ki-67 and Hoechst 33342 staining in panel (G). A representative FACS plot (left) and quantification data were shown (n=3, right).

observed a similar trend in the leukemic spleens although it is not statistically different (Figure 3A). Consistently, we also found that there was a much lower frequency of CD244-null Lin$^-$IL7-R$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγR$^+$ L-GMPs (enriched in LICs) compared to that of WT controls (*Online Supplementary Figure 4F and G*), which indicates CD244 may play a potential role in the maintenance of LICs. An *in vitro* colony-forming assay also illustrated that the clonogenic potential of CD244-null leukemia cells isolated from secondary recipients was greatly declined to 62.8%, 26.7% and 42.5% of that in WT counterparts upon first-third plating, respectively (Figure 3B), indicating

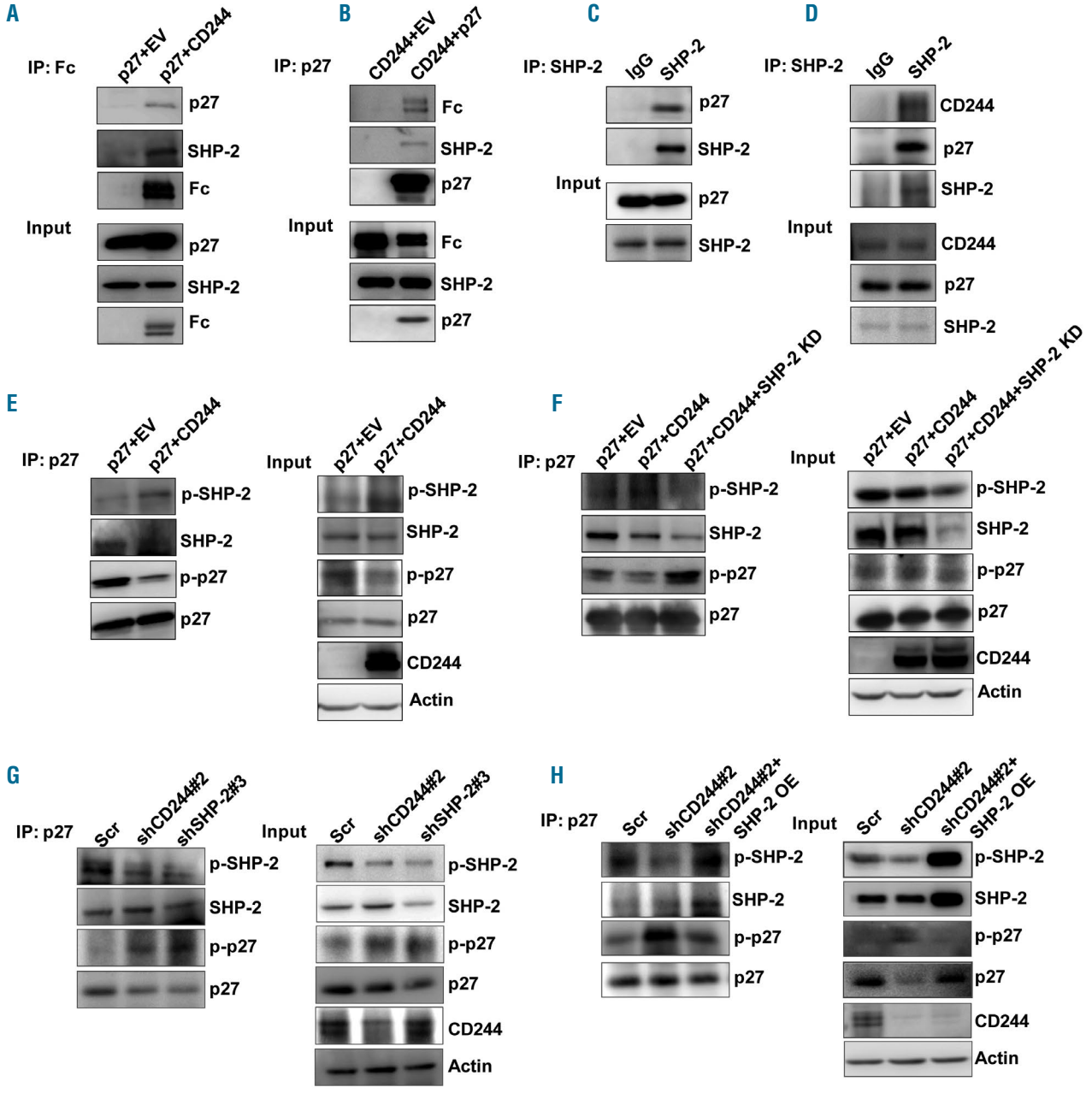

Figure 5. CD244 regulates the p27 stability through SHP-2 signaling. (A) Fc-tagged CD244 and StrepII-tagged p27 were over-expressed in 293T cells, and their lysates were immunoprecipitated by protein A beads, followed by western blotting analysis for p27 and SHP-2. (B) Fc-tagged CD244 and StrepII-tagged p27 were over-expressed in 293T cells, and their lysates were immunoprecipitated by StrepII beads, followed by western blotting analysis for Fc (CD244) and SHP-2. (C) StrepII-tagged p27 were over-expressed in 293T cells, and their lysates were immunoprecipitated by SHP-2 or IgG control antibodies, followed by western blotting analysis for p27. (D) The lysates of primary mouse AML cells were immunoprecipitated by SHP-2 or IgG control antibodies, followed by western blotting analysis for the levels of endogenous CD244 and p27. (E) Fc-tagged CD244 and StrepII-tagged p27 were over-expressed in 293T cells, and their lysates were immunoprecipitated by StrepII beads, followed by western blotting analysis for p-SHP-2, SHP-2 and 4G10 (for p-p27). (F) Fc-tagged CD244, StrepII-tagged p27 and shRNA targeting SHP-2 (shSHP-2#3) were transfected into 293T cells, and their lysates were immunoprecipitated by StrepII beads, followed by western blotting analysis for p-SHP-2, SHP-2 and 4G10 (for p-p27). The backbone empty vector (EV) was used as the control. (G) Human primary leukemia cells were infected with CD244-targeting shCD244#2, SHP-2-targeting shSHP-2#3 and scrambled shRNA. And their lysates were immunoprecipitated by p27 antibody, followed by western blotting analysis for the levels of endogenous p-SHP-2, SHP-2, 4G10 (for p-p27), p27 and CD244. (H) HEL cells were infected with scrambled shRNA, CD244-targeting shCD244#2, or CD244-targeting shCD244#2 and followed by the overexpression of SHP-2 and their lysates were immunoprecipitated by p27 antibody, followed by western blotting analysis for the levels of p-SHP-2, SHP-2, 4G10 (for p-p27), p27 and CD244.

a loss of self-renewal ability upon CD244 deletion. We then further performed a cell cycle analysis and found there was only slightly decreased G0 frequencies (representing a quiescent status) in CD244-null LICs than WT ones (9.65±0.63% *vs.* 12.75±0.79%) (Figure 3C). Although there is some evidence to show the loss of quiescence may lead to the exhaustion of LICs,[23] the connection between quiescence and stemness in LICs is still controversial since several lines of studies also imply the existence of a subset of actively cycling, non-quiescent AML cells enriched for LIC activities.[24] In addition, we also did not observe significant changes in apoptosis between WT and CD244-null LICs (Figure 3D). These results suggest that CD244 may play important roles in the proliferation (or self-renewal) rather than sustaining the quiescence or apoptosis of LICs.

CD244 manipulates p27 levels to maintain the proliferation ability of LICs

To understand the underlying mechanisms that affect the proliferation (or self-renewal) ability in CD244-null LICs, we performed quantitative RT-PCR with WT and CD244-null Mac-1$^+$/c-Kit$^+$ LICs. Taking into account the phenotypes in mouse CD244-null and human CD244-knockdown LICs, we mainly focused on the changes of molecules related to cell cycle and self-renewal, which showed that p27 was significantly down-regulated in CD244-null LICs (Figure 4A). We further evaluated p27 levels by western blotting or immunofluorescence staining and demonstrated that p27 levels were strikingly reduced in CD244-null LICs (Figure 4B and C). In addition, we also isolated cytoplasmic proteins from WT and CD244-null LICs and examined the p27 levels by western blot, which showed that there was a remarkable decrease of cytoplasmic p27, but not nuclear form of p27 (Figure 4D). Although the mRNA level of p16 was up-regulated almost 2-fold, the protein level of p16 was not significantly changed in CD244-null LICs (*Online Supplementary Figure S5A*), which indicates that p16 does not play an important role in the CD244-mediated leukemogenesis. Although it has been reported that p27 acts as a cell cycle inhibitor by interacting with CDK2 or CDK4, and may participate in sustaining the quiescent state in the MLL-leukemia cells,[25,26] p27 has also been reported to serve as cytoplasmic oncoprotein in BCR-ABL1 transformed chronic myeloid leukemia to promote cell growth and block apoptosis, rather than its effects on cyclin-dependent kinase (CDKs).[27] We also noticed that the p27 was mainly located in cytoplasm and notably reduced in CD244-null Mac-1$^+$/c-Kit$^+$ LICs (Figure 4C and D), indicating p27 may exert its additional effects (may enhance proliferation or self-renewal) on leukemia development. To confirm whether p27 is a direct downstream target for CD244, we over-expressed mouse p27 in both WT and CD244-null leukemia cells and transplanted them into the recipient mice. The mice transplanted with the p27-over-expressed CD244-null AML cells developed leukemia significantly faster than CD244-null control cells, which was comparable to WT counterparts (Figure 4E). Interestingly, the over-

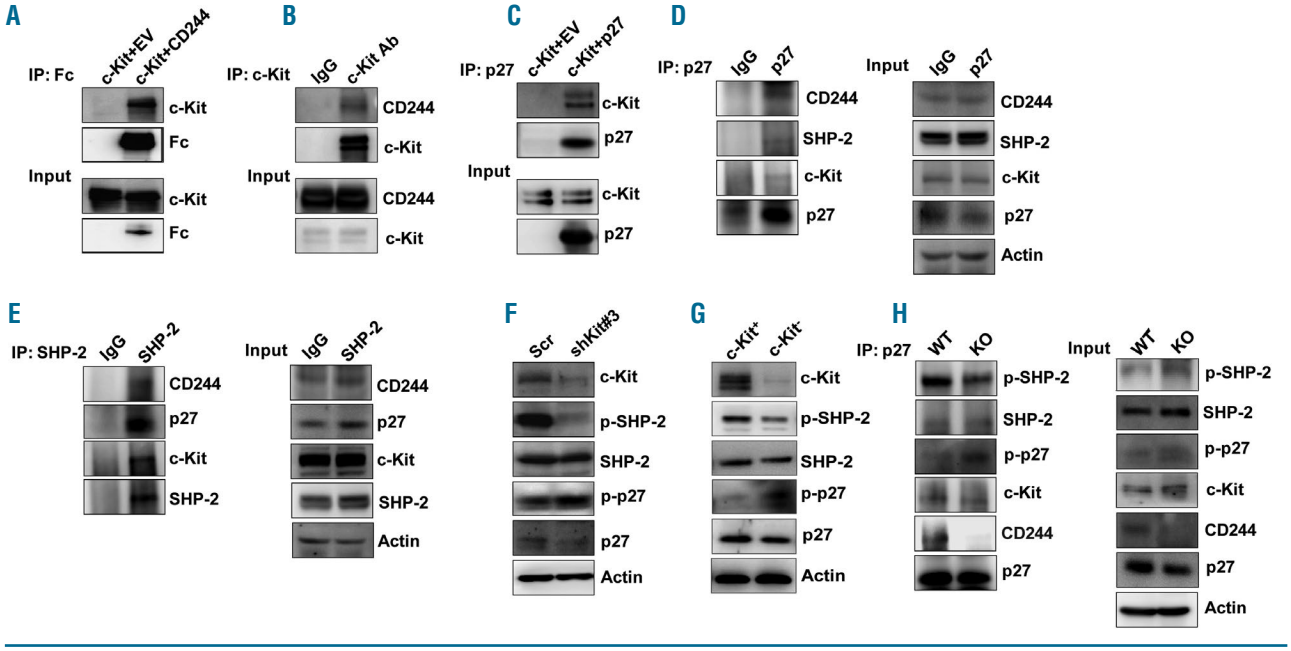

Figure 6. CD244 interacts with c-Kit to maintain the stability of p27. (A) Fc-tagged CD244 and c-Kit were over-expressed in 293T cells and their lysates were immunoprecipitated by protein A beads, followed by western blotting analysis for c-Kit. (B) Fc-tagged CD244 and c-Kit were over-expressed in 293T cells and their lysates were immunoprecipitated by c-Kit antibodies, followed by western blotting analysis for Fc (CD244). (C) StrepII-tagged p27 and c-Kit were over-expressed in 293T cells and their lysates were immunoprecipitated by StrepII beads, followed by western blotting analysis for c-Kit. (D) The lysates of HEL cells were immunoprecipitated by p27 or IgG control antibodies, followed by western blotting analysis for the levels of endogenous CD244, SHP-2 and c-Kit. (E) The lysates of HEL cells were immunoprecipitated by SHP-2 or IgG control antibodies, followed by western blotting analysis for the levels of endogenous CD244, p27 and c-Kit. (F) HEL cells transfected with shKit#3 targeting c-Kit or scrambled shRNA were used for the detection of c-Kit, p-SHP-2, SHP-2, p-p27 and p27 by western blotting analysis. (G) FACS sorted c-Kit+ and c-Kit- mouse AML cells were evaluated for the expression levels of c-Kit, p-SHP-2, SHP-2, p-p27 and p27 by western blotting analysis. (H) The lysates of WT and CD244-null primary AML cells were immunoprecipitated by p27 antibodies, followed by western blotting analysis for the levels of endogenous p-SHP-2, SHP-2, p-p27, c-Kit and CD244. IgG antibodies or the backbone empty vector (EV) was used as the control.

expression of p27 in WT leukemia cells slightly suppressed their proliferation (Figure 4E). These data also suggested that p27 may have a dose-dependent effect on leukemogenesis. The p27 levels in WT, CD244-null, p27-over-expressed WT and CD244-null AML cells after transplantation were further confirmed by western blotting (Figure 4F).

In addition, we have performed a functional assay by testing the colony forming ability *in vitro* to directly evaluate the p27 effect on the proliferation of LICs. Interestingly, the ectopic expression of p27 enhanced the proliferation of CD244-null LICs *in vitro*, but not WT ones (Figure 4G). However, high level of p27 had no impact on the cell cycle status of both WT and CD244-null leukemic blasts (Figure 4H). In contrast, we also noticed that the colonies derived from CD244-null leukemia cells appeared much smaller and more diffused compared to WT ones, which could be rescued by the p27 OE (Figure 4G), suggesting the cell division of CD244-null LICs was slowed down upon p27 deletion. In addition, we also revealed that apoptosis was not altered in CD244-null LICs (Figure 3D). Therefore, these results suggest that CD244 maintains leukemogenic potential of AML cells through p27, which may mainly contribute to the proliferation (or self-

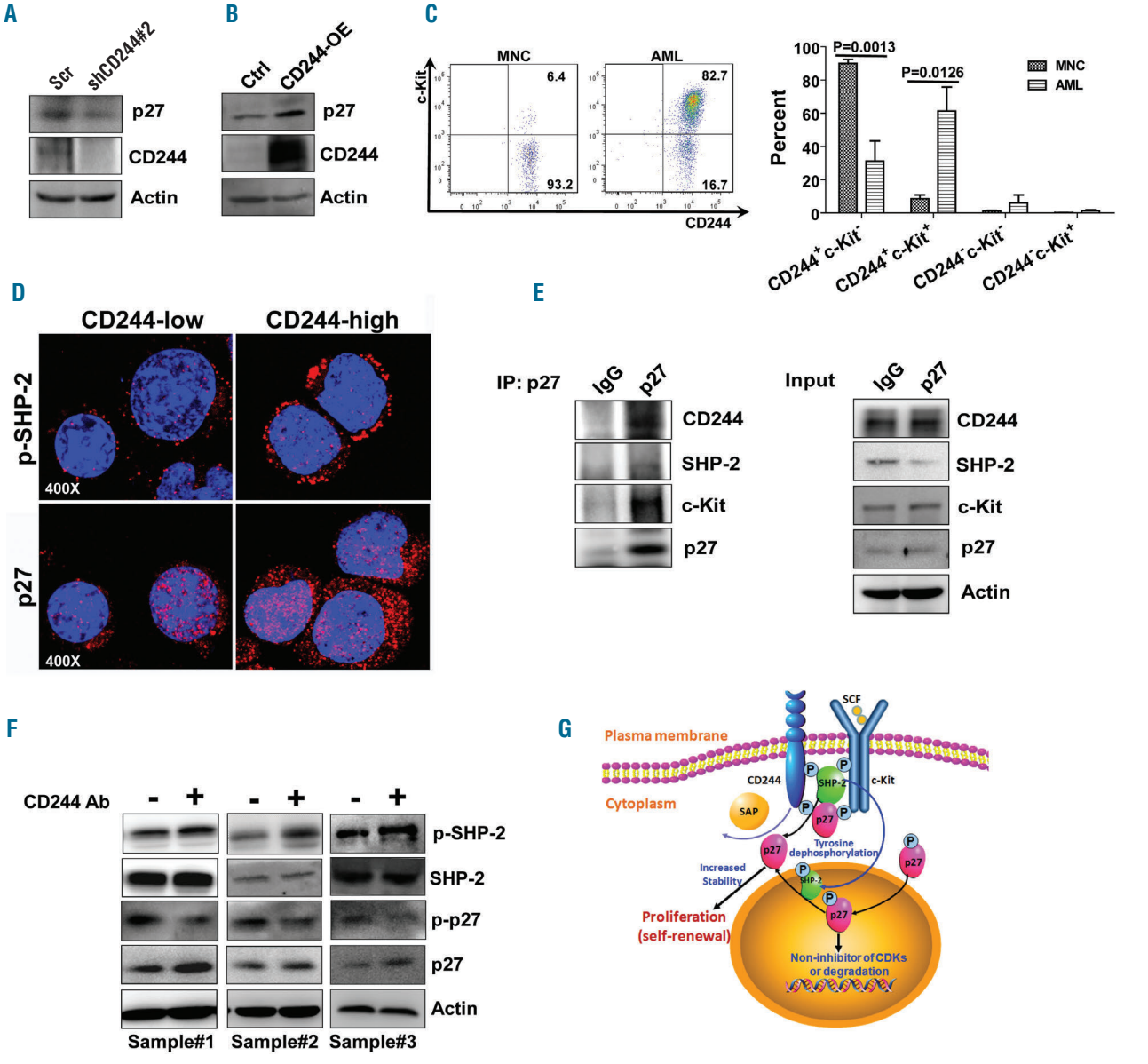

Figure 7. CD244 controls the activities of human leukemia cells through SHP-2/p27 signaling. (A) CD244 was knocked down with shCD244#2 in HEL cells, followed by western blotting analysis for p27. (B) CD244 was stably over-expressed in K562 cells, followed by western blotting analysis for p27. (C) Representative flow cytometric analysis for the expression of CD244 and c-Kit in cord blood mononuclear cells (MNC) and patients' acute myeloid leukemia (AML) cells. $CD34^+$ cells in MNC and AML samples were gated and quantified for different fractions (n=5 for MNC and n=7 for AML). (D) p-SHP-2 and p27 expression levels were examined in CD244-low and CD244-high fractions by immunofluorescence staining. (E) The lysates of human primary AML cells (type M5) were immunoprecipitated by p27 or IgG control antibodies, followed by western blotting analysis for the levels of endogenous CD244, SHP-2 and c-Kit. (F) Three human leukemia samples (type M5) were cross-linked with anti-CD244 antibodies for 24 h, and their lysates were used for the detection of p-SHP-2, SHP-2, p-p27 and p27 by western blotting analysis. (G) Working model for the roles of CD244 in leukemia initiating cells.

renewal ability), but less likely to the quiescence or apoptosis of LICs.

CD244 regulates the p27 stability through SHP-2 signaling

In consideration of the significant decrease of p27 expression levels in the CD244-null LICs, we decided to delineate how p27 is regulated by CD244. We first performed the co-immunoprecipitation assay to pull down CD244 in 293T cells and demonstrated that CD244 was associated with p27 (Figure 5A). We further examined whether the phosphatase, SHP-2, served as a mediator between CD244 and p27 since SHP-2 was known to be involved in CD244 signaling. Conceivably, SHP-2 was found to be interacted with CD244 (Figure 5A). Conversely, both CD244 and SHP-2 could also be co-immunoprecipitated by p27 (Figure 5B). p27 could also be detected when SHP-2 was pulled down from 293T cells (Figure 5C). Moreover, importantly, endogenous CD244 and p27 were associated with SHP-2 in primary mouse AML cells as determined by the co-immunoprecipitation experiment (Figure 5D).

Several studies show that tyrosine phosphorylation at Y88/Y89 of p27 leads to its conversion into a non-inhibitor of cyclin-CDK complex and is required for efficient phosphorylation on T187 by CDK2, followed by ubiquitination mediated degradation.[7,13,22] Recently, SHP-2 is reported to be a phosphatase for tyrosine dephosphorylation of p27 and can be translocated into nucleus upon stimulation and stabilizes the p27 protein levels in the cytoplasm of AML cells.[26,28] Indeed, overexpression of CD244 could dramatically up-regulate the phosphorylation of SHP-2 which substantially dephosphorylated p27 (Figure 5E). To reveal whether SHP-2 is a direct player for CD244 to dephosphorylate p27, we knocked down SHP-2 with a validated shSHP-2#3 (*Online Supplementary Figure S5B*) in CD244-over-expressed 293T cells and examined the phospho-p27 levels upon co-immunoprecipitation. Indeed, phoshpo-p27 was highly up-regulated after SHP-2 deletion (Figure 5F). Furthermore, we have performed the experiment in human primary AML cells to confirm the regulatory role of SHP-2. We demonstrated that the p-p27 was significantly elevated upon SHP-2 or CD244 knockdown in human primary AML cells (Figure 5G and *Online Supplementary Figure S5C*). Then we continued to knock down CD244 in HEL cells to examine the regulatory role of SHP-2. Interestingly, we did find that p-SHP-2 and p27 were notably reduced, which could be rescued by over-expressing SHP-2 in CD244-knockdown HEL cells (Figure 5H). Taken together, CD244 is associated with SHP-2 to down-regulate the phosphorylation of p27, which further sustains its stability.

CD244 interacts with c-Kit to maintain the stability of p27

c-Kit has been reported as a critical regulator for both HSCs and AML initiation through SHP-2 or SCL pathway. Constitutively, activation of c-Kit may lead to the marked acceleration of leukemia development.[29-34] Regarding the significant decrease of c-Kit$^+$ LICs in CD244-null leukemic mice, we speculate that there is a connection between CD244, c-Kit and p27. Therefore, we conducted a co-immunoprecipitation experiment with overexpression of both Fc-tagged CD244 and c-Kit in 293T cells, followed by pull-down with either protein A beads or anti-c-Kit antibodies. CD244 was indeed associated with c-Kit (Figure 6A and B). We also provided further evidence to show that p27 was directly interacted with c-Kit when p27 was pulled down from 293T cells (Figure 6C). Consistently, we further confirmed the interactions of CD244, SHP-2 and c-Kit in leukemia cell line, HEL cells, which is an erythroid leukemia cell line expressing both CD244 and c-Kit (Figure 6D and E).

Recently, Marcelo *et al.* reported that c-Kit promoted the expression of p27 in hemogenic endothelial cells,[35] which led us to further evaluate the relationship of c-Kit and p27 in leukemia cells. By using shKit#3 specifically targeting c-Kit (*Online Supplementary Figure S5D*), we knocked down c-Kit expression in HEL cells and demonstrated that knockdown of c-Kit could efficiently down-regulate phosphorylation of SHP-2 and total p27 expression levels, but enhance the p-p27 level (Figure 6F). Because HEL cells were JAK2 V617F positive and may exert artificial effect on SHP-2/p27 signaling, we have further performed the experiment related to the role of c-Kit on primary mouse AML cells. As shown in Figure 6G, we examined the c-Kit/SHP-2/p27 pathways in c-Kit$^+$ or c-Kit$^-$ mouse AML cells and demonstrated that c-Kit level indeed enhances the SHP-2 signaling to decrease the phosphorylation level of p27 to maintain the stability of total p27 expression.

Next, we pulled down p27 with the antibody specifically against p27 in WT and CD244-null AML cells, and revealed c-Kit, SHP-2 and CD244 were indeed directly associated with p27 in physiological conditions (Figure 6H and *Online Supplementary Figure S5E*). Phospho-SHP-2 level was also significantly decreased, which was concordant with increased level of phospho-p27 (Figure 6H and *Online Supplementary Figure S5E*). Importantly, the interactions between these three proteins were CD244-dependent since there were much lower levels of p-SHP-2 and c-Kit, or higher levels of p-p27 in CD244-null leukemia cells (Figure 6H and *Online Supplementary Figure S5E*). Although we found no significant difference in the phospho-SHP-2 level in the total lysate of WT and CD244-null AML cells, there was a remarkable decreased phospho-SHP-2 level in CD244-null AML cells when p27 was immunoprecipitated, indicating that only the p27-interacting form of SHP-2 was affected upon CD244 deletion (Figure 6H and *Online Supplementary Figure S5E*). These results suggest that CD244 might co-ordinate with c-Kit to regulate the levels of p27 through SHP-2 pathway to maintain the proliferation ability of LICs.

CD244 controls the proliferation of human leukemia cells through SHP-2/p27 signaling

To tease apart the CD244/SHP-2/p27 signaling in human leukemia cells, we examined the p27 levels in either CD244-silenced HEL cells or CD244-over-expressed K562 cells (expressing very low levels of CD244) by immunoblotting, which showed that p27 levels were also consistently down-regulated or up-regulated, respectively (Figure 7A and B). We then measured the expression levels of CD244 and c-Kit in CD34$^+$ cells (enriched for LICs) from several AML patients and demonstrated that 61.3% of CD34$^+$/CD244$^+$ leukemia cells were co-expressed with c-Kit, which was much higher than cord blood CD34$^+$/CD244$^+$ mononuclear cells (MNC) (61.31±14.42% *vs.* 8.61±2.25%) (Figure 7C). These results indicate that CD244 and c-Kit may also function synergistically in human LICs. We further isolated CD244-low and CD244-

high leukemia cells and examined the phospho-SHP-2 and p27 level by immunofluorescence staining. The phospho-SHP-2 and p27 levels were much higher in the cytoplasm of CD244-high cells (Figure 7D).

We next examined the CD244/c-Kit/SHP-2/p27 signaling in human primary AML cells and revealed similar interactions among these molecules as in a mouse leukemia model, as analyzed by the co-immunoprecipitation experiments (Figure 7E). Furthermore, CD244 receptors of human $CD34^+$ LICs were cross-linked with the functional anti-CD244 antibodies to test whether the downstream SHP-2/p27 signaling could be efficiently induced. In line with the findings in mouse leukemia cells, phospho-SHP-2 and p27 levels were indeed increased, while p-p27 was consistently decreased upon the antibody stimulation in all the 3 tested human samples (Figure 7F). In summary, a working model is shown in Figure 7G, and our findings show that CD244 collaborates with c-Kit to regulate SHP-2 phosphatase activity to dephosphorylate and stabilize p27 in cytoplasm for maintaining the proliferation (self-renewal) ability of LICs.

Discussion

In this study, we characterized CD244 function in AML initiating cells and provided solid evidence that CD244 has a differential effect on LICs compared to normal HSCs. We further revealed CD244 might co-operate with c-Kit to mediate its downstream signaling through SHP-2/p27 to regulate the proliferation (self-renewal) of LICs. To our knowledge, this is the first body of evidence showing that CD244 is critical for leukemia development. These results consolidated our previous hypothesis that immune inhibitory receptors are involved in maintenance of the stemness of HSCs and LICs. Intriguingly, our study also indicated that both ITIM and ITSM containing immune molecules may be essential for different types of leukemia.

Our previous findings suggested that many ITIM-containing immune molecules recruit SHP-2, SHP-1 or SHIP1 to initiate numerous downstream signaling pathways. Our current study also indicates that CD244 is also associated with SHP-2 as well as p27 to control leukemia development. Nonetheless, how CD244 exerts its effect on SHP-2 and p27 needs further clarification. Our data showed that CD244 could up-regulate p27 levels either through transactivation or post-translational modification. However, it is still not clear which pathway plays the dominant roles in leukemogenesis and how these dual functions are integrated. Meanwhile, how CD244 transactivates p27 is still a mystery. p27 is known to be important to maintain the repopulation ability and quiescence of HSCs by co-operating with p57.[36,37] p27 has also been found to play differential roles in different types of cells, including acting as a cell cycle inhibitor to maintain the quiescent status of LICs[25] and enhancing the migration of mouse embryonic fibroblasts.[38] Nevertheless, several studies claimed that LICs existed in a non-quiescent population of cells controlled by certain cyclins, such as cyclin D2.[39] Iwasaki *et al*. also demonstrated that CD93 marks a non-quiescent human LIC population and regulates LIC self-renewal predominantly by silencing CDKN2B.[24] Herein, we also found CD244 may sustain the proliferation ability of LICs by stabilizing p27 levels independent of cell cycle inhibition, suggesting cell cycle regulators play differential roles in normal or cancer stem cells, which is critical for the development of new strategies for cancer treatment.

Furthermore, our findings indicate that c-Kit may interact with CD244 to regulate the expression levels of p27. However, there are several intriguing questions that remain to be addressed. 1) Which domain of c-Kit interplays with CD244: the extra-cellular or intra-cellular domain? 2) How does c-Kit stabilize p27 levels through CD244? 3) Does CD244 up-regulate the expression of c-Kit? 4) Although it is well known that c-Kit has an important function in mouse LIC maintenance, its role in human LICs and related mechanisms remains elusive. We are currently in the process of investigating these issues to pinpoint the CD244/c-Kit/SHP-2/p27 signaling in LICs.

Our study indicates that CD244 is highly expressed on different types of AML, but may not be expressed on B-cell ALL, which suggests that the expression of CD244 may serve as a threshold for myeloid differentiation. Whether there are any other specific regulators controlling CD244 expression in LICs needs to be clarified. Determining the novel mechanisms controlling the expression and activation of CD244 will open a new avenue for treatment of AML. In conclusion, our results provide strong evidence that CD244 co-operates with c-Kit to regulate leukemogenesis through SHP-2/p27 signaling.

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