Title

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Journal

Molecular Oncology, 7(6)

ISSN

1574-7891

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Publication Date

2013-12-01

DOI

10.1016/j.molonc.2013.07.011

Peer reviewed

MOLECULAR ONCOLOGY 7 (2013) 1069–1082

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/molonc

p27^{kip1} maintains a subset of leukemia stem cells in the quiescent state in murine MLL-leukemia

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ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form
22 July 2013
Accepted 31 July 2013
Available online 20 August 2013

Keywords:
MLL-leukemia
p27^{kip1}
Leukemia stem cell
Quiescence

ABSTRACT

MLL (mixed-lineage leukemia)-fusion genes induce the development of leukemia through deregulation of normal MLL target genes, such as *HOXA9 and MEIS1*. Both *HOXA9* and *MEIS1* are required for MLL-fusion gene-induced leukemogenesis. Co-expression of *HOXA9* and *MEIS1* induces acute myeloid leukemia (AML) similar to that seen in mice in which MLL-fusion genes are over-expressed. p27^{kip1} (p27 hereafter), a negative regulator of the cell cycle, has also been defined as an MLL target, the expression of which is up-regulated in MLL leukemic cells (LCs). To investigate whether p27 plays a role in the pathogenesis of MLL-leukemia, we examined the effects of *p27* deletion ($p27^{-/-}$) on MLL-AF9 (MA9)-induced murine AML development. HOXA9/MEIS1 (H/M)-induced, *p27* wild-type ($p27^{+/+}$) and $p27^{-/-}$ AML were studied in parallel as controls. We found that LCs from both MA9-AML and H/M-AML can be separated into three fractions, a CD117^{-}CD11b^{hi} differentiated fraction as well as CD117^{+}CD11b^{hi} and CD117^{+}CD11b^{lo}, two less differentiated fractions. The CD117^{+}CD11b^{lo} fraction, comprising only 1–3% of total LCs, expresses higher levels of early hematopoietic progenitor markers but lower levels of mature myeloid cell markers compared to other populations of LCs. p27 is expressed and is required for maintaining the quiescent and drug-resistant states of the CD117^{+}CD11b^{lo} fraction of MA9-LCs but not of H/M-LCs. *p27* deletion significantly compromises the leukemogenic capacity of CD117^{+}CD11b^{lo} MA9-LCs by reducing the frequency of leukemic stem cells (LSCs) but does not do so in H/M-LCs. In addition, we found that p27 is highly expressed and required for cell cycle arrest in the CD117^{-}CD11b^{hi} fraction in both types of LCs. Furthermore, we

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1574-7891/$ – see front matter Published by Elsevier B.V. on behalf of Federation of European Biochemical Societies.
http://dx.doi.org/10.1016/j.molonc.2013.07.011

found that *c-Myc* expression is required for maintaining LCs in an undifferentiated state independently of proliferation. We concluded that p27 represses the proliferation of LCs, which is specifically required for maintaining the quiescent and drug-resistant states of a small subset of MA9-LSCs in collaboration with the differentiation blockage function of c-Myc.

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1. Introduction

MLL protein, a large histone methyltransferase, maintains the expression of a subset of genes by specifically methylating histone H3 at lysine 4 (H3K4) in the promoter regions of its target genes (Milne et al., 2002). Most MLL-target genes, such as those within the *Hox* loci and many other genes, are master regulators of development and are also key regulators of hematopoiesis (Abramovich and Humphries, 2005). Gene knockout studies suggested that Mll is dispensable for the initiation of stage-specific expression of its target *Hox* genes but is absolutely required for the maintenance of expression of such genes during early development (Yu et al., 1995). Mice with homozygous *Mll* mutation suffer early embryonic lethality with significant defects in both yolk sac primary and AGM definitive hematopoiesis (Yagi et al., 1998; Hess et al., 1997; Ernst et al., 2004a,b). The self-renewal and proliferation of hematopoietic stem cells (HSCs) are significantly compromised by Mll inactivation (McMahon et al., 2007; Jude et al., 2007). The hematopoietic defects in *Mll*-knockout mice ($Mll^{-/-}$) might be mainly due to a reduction in the expression of *Hox* genes because the proliferative defects of hematopoietic stem and progenitor cells (HSPCs) derived from $Mll^{-/-}$ embryonic stem cells can be largely rescued by the overexpression of *Hox*-cluster genes (Ernst et al., 2004a,b).

MLL-rearrangements are found in >70% of infant acute lymphoblastic leukemias (ALL), approximately 10% adult acute myeloid leukemias (AMLs) and >30% of therapy-related leukemias (Liu et al., 2009). MLL-fusion proteins, the products of *MLL* rearrangements, induce the development of leukemia through deregulating MLL target genes, such as *HOXA9*, *HOXA7*, *MEIS1* and *PBX1*. (Marschalek, 2011; Muntean and Hess, 2011; Faber et al., 2009; Dou and Hess, 2008; Zeisig et al., 2004; Kroon et al., 1998; Li et al., 2009; Andreeff et al., 2008). Although they lack histone methyltransferase activity, MLL-fusion proteins contain Menin/LEDGF binding sites, a nuclear localization sequence and a CxxC motif, all of which are features essential for the correct targeting of the MLL complex to the promoter regions of its target genes (Milne et al., 2010; Milne et al., 2005; Zeleznik-Le et al., 1994; Ayton et al., 2004). Interrupting the DNA binding ability of MLL-fusion proteins by disrupting these DNA binding motifs will significantly compromise their leukemogenic capacity due to their consequent failure to up-regulate MLL target genes (Slany et al., 1998; Cierpicki et al., 2010; Yokoyama et al., 2005; Caslini et al., 2007; Yokoyama and Cleary, 2008; Bach et al., 2009). The requirement for the wild-type (*WT*) allele of MLL for MLL-fusion protein-induced leukemic transformation suggested that both MLL-fusion protein and WT-MLL might be recruited to the regulatory regions of target genes. An MLL-fusion protein might be dependent upon WT-MLL's methyltransferase activity and hijack the normal transcription/elongation machinery to induce target gene expression (Milne et al., 2010; Muntean et al., 2010; Bernt et al., 2011; Chang et al., 2010; Thiel et al., 2011). Many MLL target genes are consistently up-regulated in human MLL cells (Slany, 2009; Imamura et al., 2002; Lawrence et al., 1999; Armstrong et al., 2002). Several of these genes, including *HOXA9*, *HOXA7*, *MEIS1* and *PBX1/2*, have been shown to be required for MLL-fusion protein-induced leukemia development as shown by genetic studies (Faber et al., 2009; Wong et al., 2007; Kumar et al., 2009; Ayton and Cleary, 2003). In addition, co-expression of HOXA9 and MEIS1 induces leukemic transformation in HSCs by promoting self-renewal/proliferation and blocking differentiation, which mimic most of the functions of MLL-fusion proteins (Zeisig et al., 2004; Kroon et al., 1998). These studies demonstrated the key role of *Hox*-cluster genes in the pathogenesis of MLL-fusion protein-induced leukemia.

$p27^{kip1}$ (p27 hereafter), a negative regulator of the cell cycle, has been identified as a direct target of MLL (Milne et al., 2005; Xia et al., 2005). Menin, the product of the tumor suppressor gene *Multiple endocrine neoplasia*, represses cell growth by inducing p27 expression (Horiguchi et al., 2009). It was found that Menin functions as an adaptor for the interface of MLL and LEDGF (lens epithelium-derived growth factor), which mediates the specific binding of MLL to chromatin and DNA (Milne et al., 2005; Caslini et al., 2007; Yokoyama and Cleary, 2008). Both Menin and LEDGF are required for the correct localization of MLL to its target genes and inducing target gene expression. Genetic disruption of the MLL/Menin/LEDGF interaction leads to down-regulation of MLL target gene expression in MLL-fusion gene-transduced cells and blocks the development of leukemia (Yokoyama et al., 2005; Caslini et al., 2007; Yokoyama and Cleary, 2008). In MLL-fusion gene-transduced LCs, Menin recruits both WT MLL and MLL-fusion proteins to the p27 promoter, which might cooperatively up-regulate p27 expression (Milne et al., 2005; Xia et al., 2005). In contrast to other leukemogenic fusion protein-related LCs, Flt3-L (Flt3-Ligand) specifically induces p27 expression in MLL-fusion-related LCs and represses their proliferation (Furuichi et al., 2007). This specific up-regulation of p27 in MLL LCs suggested that it might play a distinct role in the pathogenesis of MLL-fusion gene-induced leukemia.

To investigate the role of p27 in the development of MLL-fusion gene-induced leukemia, we evaluated the effects of deletion of the *p27* gene on the pathogenesis of MLL leukemia by comparative study of the *in vitro* growth behaviors and *in vivo* leukemogenic activity of $p27^{+/+}$ (p27 WT) and $p27^{-/-}$ (p27-knockout), MA9 (MLL-AF9) murine LCs, as well as $p27^{+/+}$ and $p27^{-/-}$ H/M (HOXA9/MEIS1) murine LCs. We found that in

both types of leukemia, p27 is highly expressed in $CD117^-$ differentiated LCs, and this factor is responsible for the cell cycle arrest state of these cells. However, in $CD117^+$ undifferentiated LCs, p27 is expressed only in the $CD11b^{lo}$ population of MA9-LCs but not in the same population of H/M-LCs. The $CD117^+CD11b^{lo}$ population represents a small subset of LCs (1–3%) in both types of leukemia that express several markers specific for HSPCs. Interestingly, *p27* expression can be induced by Flt3-L and SCF in $CD117^+$ MA9-LCs but not in $CD117^+$ H/M-LCs. Moreover, we found that p27 is responsible for maintaining quiescence, leukemogenic ability and drug-resistance in the $CD117^+CD11b^{lo}$ population of MA9-LCs but not in H/M LCs.

2. Materials and methods

2.1. Mice

All experiments were performed in strict accordance with the provisions of Loyola University Chicago IACUC protocol #06-013. $p27^{-/-}$ mice (C57Bl6J background, which are $CD45.2^+$, Stock Number: 010834) and Ptprc recipient mice (C57Bl6J background, which are $CD45.1^+$, Stock Number: 002725) were purchased from the Jackson Laboratory and maintained in the Department of Comparative Medicine, Loyola University Medical Center. *c-Myc*$^{fx/fx}$ conditional *c-Myc* knockout mice (de Alboran et al., 2001) were kindly provided by Dr. Frederick W. Alt of the Howard Hughes Medical Institute (C57Bl6J background, $CD45.2^+$). *c-Myc*$^{fx/fx}$*p27*$^{-/-}$ compound-mutant mice were generated by crossing $p27^{-/-}$ mice with *c-Myc*$^{fx/fx}$ mice. Genotyping for $p27^{-/-}$ and *c-Myc*$^{fx/fx}$ mice was performed by tail biopsy using PCR assay with the following primers: p27 forward: GAACTAACCCGGGACTTGG AGAAGC; p27 reverse: TAACCCA GCCTGATTGTCTGACGAG; c-Myc forward: GCCCCTGAATTGCTAGGAAGACTG; c-Myc reverse: CCGACCGGGTCCGAGTCC CTATT.

2.2. $CD117^+$ HSPC isolation, infection and in vitro culture

HoxA9-GFP (Cat. No. 20977) and Meis1-YFP (Cat. No. 21013) plasmids were purchased from *Addgene*. MLL-AF9 (MA9), HoxA9 or Meis1-expressing retroviruses were produced by co-transfecting 293T-HEK cells with packaging plasmids and the appropriate retroviral vector (MLL-AF9 (MA9)-neo, HoxA9-GFP or Meis1-YFP) using *Lipofectamine 2000*® reagent (*Invitrogen*). Bone marrow (BM) $CD117^+$ HSPCs from $p27^{+/+}$ and $p27^{-/-}$ mice were enriched using *EasySep*® Mouse CD117 Positive Selection Kit (*StemCell Technologies*) and cultured in 12-well plates in 4-cytokine stem cell culture medium (RPMI 1640 medium containing 10% FBS, 100 ng/ml SCF, 50 ng/ml IL-6, 10 ng/ml IL-3, and 20 ng/ml GM-CSF) overnight to induce cell proliferation (pre-stimulation) in order to improve the efficiency of infection. Pre-stimulated HSPCs were infected with MA9-expressing retrovirus or co-infected with HoxA9 or Meis1-expressing retrovirus by spinoculation in the presence of 4 μg/mL polybrene. Twenty-four hours after the spinoculation, MA9-infected cells were selected with 1.2 mg/ml G418 for 2 weeks with daily medium change, whereas HoxA9 and Meis1 co-infected cells were purified by sorting for GFP^+/YFP^+ cells using FACS and continuously culturing for two weeks. HoxA9 and Meis1 co-infected cells were analyzed by flow cytometry to confirm their purity. Samples with >99% GFP^+/YFP^+ cells were used for further studies. All cytokines used in this study were purchased from *eBioscience*.

2.3. In vitro *colony-forming assay*

Indicated numbers of pre-LCs or LCs were seeded into *MethoCult*® GF M3434 medium (*StemCell Technologies*) and incubated at 37 °C., 100% humidity, and 5% CO_2. Numbers of colonies were counted on day 7 of culturing.

2.4. In vivo *transplantation and leukemogenic assay*

Recipient mice were lethally irradiated (10.5 Gy) in a Nordion *Gammacell 40* irradiator. Indicated numbers of pre-LCs or LCs ($CD45.2^+$) were mixed with normal support BM mononuclear cells (MNCs, $CD45.1^+$) and transplanted into recipient mice ($CD45.1^+$). Mice were monitored for signs of leukemia. Leukemia was diagnosed by the detection of leukemic blasts in PB and BM, as well as by observing liver infiltration. The donor origin of the leukemic cells was confirmed by CD45.2 staining for MA9 leukemia or GFP^+/YFP^+ for H/M leukemia. Immunophenotypes of LCs were examined by staining the cells with fluorescein-conjugated antibodies followed by flow cytometric analysis. Antibodies used in this study included: rat anti-mouse CD117, CD11b, CD34, CD123, CD135, CD48, CD150, CD19, Ter119, CD11c, F4/80 and Gr1, all of which were purchased from *eBioscience*. After mice were sacrificed, their livers were immediately collected. After 2–3 days fixation in zinc formalin, liver tissues were embedded in paraffin. Sections were cut for H & E staining.

2.5. *BrdU incorporation*

To examine the proliferation of $CD117^-CD11b^{hi}$, $CD117^+CD11b^{hi}$ and $CD117^+CD11b^{lo}$ LCs, 4 h prior to being sacrificed, mice were injected with BrdU at 50 μg/g body weight. MNCs were collected from leukemic mice. The percentages of $BrdU^+$ cells within different subsets of LCs were assessed by CD117 and CD11b cell-surface marker staining followed by cell permeabilization and BrdU antibody staining, as previously reported (Zhang et al., 2003). To examine the proliferation of pre-LCs and LCs cultured *in vitro*, cells were treated with BrdU at a concentration of 10 ng/ml commencing 1–2 h prior to collection. The percentages of $BrdU^+$ cells were detected by cell permeabilization and BrdU antibody staining.

2.6. *Cell cycle analysis*

$CD117^-CD11b^{hi}$, $CD117^+CD11b^{hi}$ and $CD117^+CD11b^{lo}$ LCs were sorted from the spleens of leukemic mice by FACS and fixed in cold 70% ethanol for 3 h. Cells were then washed twice with PBS containing 0.5 μg/ml pyronin-Y and 2 μg/ml Hoechst 33342 and stained in the same solution for 10 min. Data were collected using a *FACSAria*® cytometer (*BD Bioscience*) and analyzed using *FlowJo*® software.

2.7. Annexin-V staining to assess apoptosis

Cells were stained with allophycocyanin-conjugated Annexin-V in binding buffer following the manufacturer's instructions (*BD Biosciences*). Death of cells was determined by examination of the percentage of Annexin-V^+ cells by flow cytometry.

2.8. qRT-PCR

RNA was isolated using *RNeasy Plus*® Mini kit (*Qiagen*). cDNA was prepared using *SuperScript*® First-Strand Synthesis System (*Invitrogen*). Quantitative PCR for detecting *p27*, *p21* and *p57* mRNA expression levels was conducted using *SYBR Green PCR Master*® (*Applied Biosystems*). Information concerning the primers used can be found in Supplementary Table 1. Triplicate RT-PCRs were performed.

2.9. Statistical analyses

The serial dilution and competitive transplantation data were analyzed by *L-Calc*™ Software (*StemCell Technologies*). All other data were analyzed using the 2-tailed Student *t* Test with *GraphPad Prism*® software (Version 5.04) to identify significant differences between groups. Values are expressed as means ± SEM. Differences were regarded as significant at $p < 0.05$.

3. Results

3.1. p27 deletion does not affect the immunophenotype of MA9-AML and H/M-AML LCs in mice

To study whether *p27* is required for the pathogenesis of MA9-AML, we generated both $p27^{+/+}$ and $p27^{-/-}$ murine MA9-AMLs by transplanting MA9-transduced $p27^{+/+}$ and $p27^{-/-}$ HSPCs ($CD45.2^+$) into lethally-irradiated congenic mice ($CD45.1^+$) with the support of $CD45.1^+$ normal BM-MNCs. We also generated $p27^{+/+}$ and $p27^{-/-}$ murine H/M-AMLs by transplanting H/M-transduced $p27^{+/+}$ and $p27^{-/-}$ HSPCs into lethally-irradiated congenic mice. The recipient mice were monitored for signs of leukemia by examining their peripheral blood (PB) for the

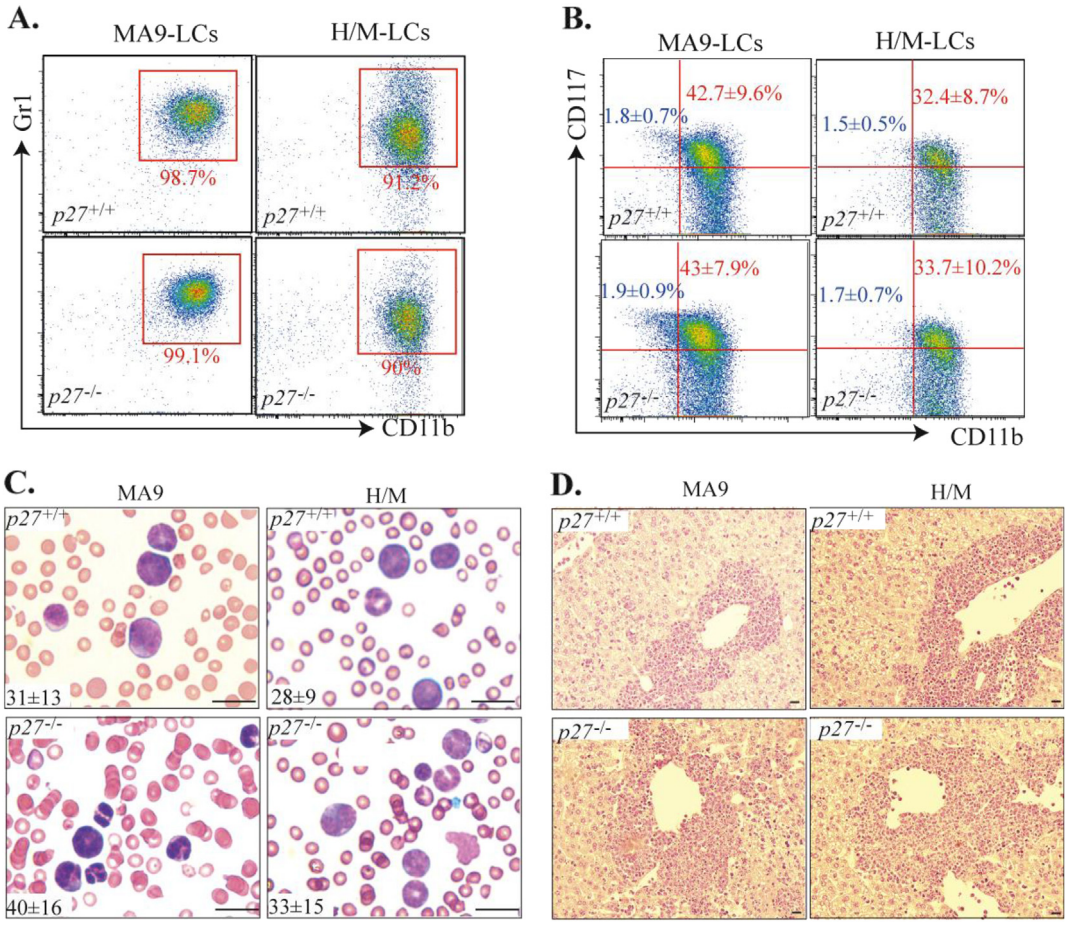

Figure 1 – p27 deletion does not affect the phenotype of either MA9- or H/M-induced leukemia. Mice which had received MA9-transduced $p27^{+/+}$ or $p27^{-/-}$ HPSCs as well as mice which had received H/M-transduced $p27^{+/+}$ or $p27^{-/-}$ HPSCs were monitored for leukemia development. After leukemia developed, mice were sacrificed. LCs were collected from the spleens of leukemic mice and stained with Gr1, CD11b and CD117 antibodies. Immunophenotype was compared between $p27^{+/+}$ and $p27^{-/-}$ MA9-LCs as well as between $p27^{+/+}$ and $p27^{-/-}$ H/M-LCs (A & B). Percentages of $CD117^+$ and $CD117^+CD11b^{lo}$ cells are shown in red and blue, respectively (B). Leukemia was verified by peripheral blood smear (C) and liver histologic section studies (D). Average numbers (× 10^6/ml) of white blood cell counts (WBC) are shown at the lower left corner of each picture (C). $n = 6$. Bars represent 50 μm in C and 100 μm in D.

percentage of CD45.2$^+$ LCs (for MA9 transduction) and the percentage of GFP$^+$YFP$^+$ LCs (for H/M transduction). After leukemia developed (indicated by LCs > 60% in PB), mice were sacrificed and LCs from the BM and spleens of the mice were collected for phenotype analysis. We found that p27 deletion delayed MA9-leukemia development but had no significant influence on H/M leukemia development as shown by the survival curves of the recipient mice (Supplementary Figure 1). In addition, we found that almost all LCs derived from both MA9- and H/M-transduced HSPCs were CD11b$^+$Gr1$^+$ myeloid blasts which were not affected by the deletion of *p27* (Figure 1A). Consistent with previous studies (Somervaille and Cleary, 2006), we found that 35–60% (average 43%) of MA9-LCs and 21–46% (average 32%) of H/M-LCs also express moderate levels of CD117 (CD117$^+$). Again here, these percentages were not affected by *p27* deletion (Figure 1B). In addition, we found that CD117$^+$ LCs in both types of leukemia can be further separated into CD11b^{hi} and CD11b^{lo} populations. The percentage of CD117$^+$CD11b^{lo} cells is 1–3% in both MA9-LCs and H/M-LCs, which was not affected by *p27* deletion (Figure 1B). Furthermore, no significant differences in LCs in PB (Figure 1C) or liver infiltrates (Figure 1D) were observed between mice receiving MA9-transduced *p27*$^{+/+}$ and *p27*$^{-/-}$ HPSCs, nor between mice receiving H/M-transduced *p27*$^{+/+}$ and *p27*$^{-/-}$ HSPCs. These data indicated that p27 is not required for the leukemic transformation of either MA9-transduced HSPCs or H/M-transduced HSPCs.

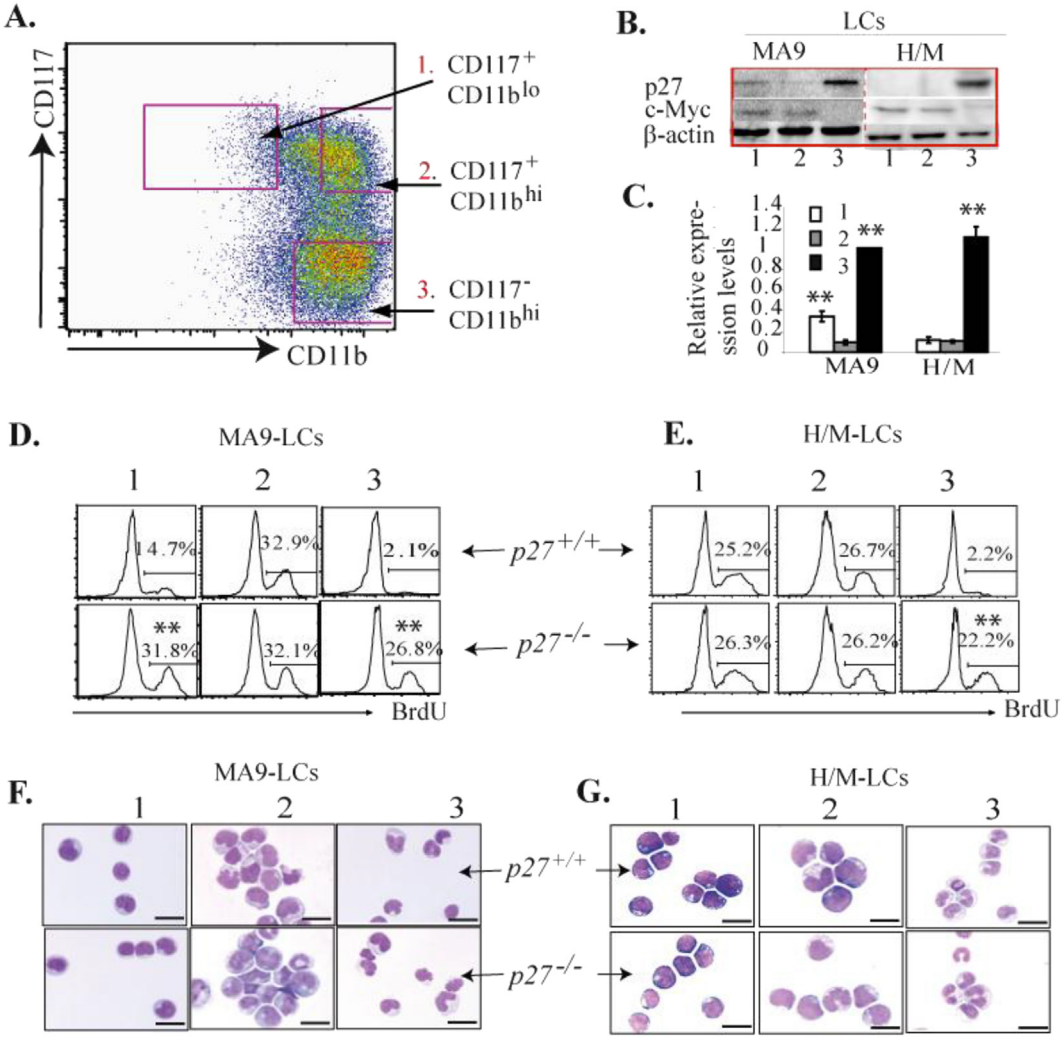

Figure 2 – p27 is required for maintaining the quiescent state of the CD117$^+$CD11b^{lo} population of MA9-LCs but not of H/M-LCs. A–C. LCs were collected from the spleens of *p27*$^{+/+}$ MA9 (CD45.2$^+$) and *p27*$^{+/+}$ H/M (GFP$^+$/YFP$^+$) leukemic mice and were separated into CD117$^+$CD11b^{lo}, CD117$^+$CD11b^{hi} and CD117$^-$CD11b^{hi} populations by FACS (A). p27 and c-Myc expression in these populations of cells was examined by Western Blotting (B) and p27 expression by qRT-PCR assays (C). The relative levels of mRNA expression in C were first normalized to the expression levels of β-actin in each sample and then further normalized by relative expression levels of *p27* in the CD117$^-$CD11b^{hi} population of MA9-LCs. ** indicates significant increase compared to CD117$^+$CD11b^{hi} groups ($p < 0.01$). D & E. Leukemic mice were injected with 1 mg BrdU 2 h prior to being sacrificed. Proliferation of CD117$^+$CD11b^{lo}, CD117$^+$CD11b^{hi} and CD117$^-$CD11b^{hi} populations of LCs was compared between *p27*$^{+/+}$ and *p27*$^{-/-}$ MA9-LCs (D) as well as between *p27*$^{+/+}$ and *p27*$^{-/-}$ H/M-LCs (E). ** indicates significant increase compared to corresponding population in *p27*$^{+/+}$ LCs ($p < 0.01$). Comparison of the morphology of the three populations in both *p27*$^{+/+}$ and *p27*$^{-/-}$ MA9-LCs (F) as well as between *p27*$^{+/+}$ and *p27*$^{-/-}$ H/M-LCs (G). Bars represent 50 μm.

3.2. p27 is required for maintaining the quiescent state of the CD117^{+}CD11b^{lo} population of MA9-LCs but not of H/M-LCs

It has been reported that the *p27* gene is a direct target of MLL and MLL-fusion proteins, the expression of which is up-regulated in MLL LCs but not in other types of LCs (Milne et al., 2005; Xia et al., 2005; Furuichi et al., 2007). However, we found that levels of p27 were comparable between MA9-LCs and H/M-LCs when an unsorted population of cells was examined (Supplementary Figure 2). Interestingly, we found that in MA9-LCs, p27 is expressed in the CD117^{+}CD11b^{lo} population and is down-regulated in the CD117^{+}CD11b^{hi} population, and is significantly up-regulated in CD117^{-}CD11b^{hi} population, while in H/M-LCs, the expression levels of p27 are low in both the CD117^{+}CD11b^{lo} and CD117^{+}CD11b^{hi} populations, and is also up-regulated in the CD117^{-}CD11b^{hi} population (Figure 2A–C). However, the expression of p21^{cip1} (p21 hereafter) and p57^{kip2} (p57 hereafter), which are other cell cycle inhibitory proteins in the p27 family, as well as the expression of p18^{ink4c}, are comparable between CD117^{+}CD11b^{lo} and CD117^{+}CD11b^{hi} populations in both types of LCs, while p15^{ink4b} and p16 ^{ink4b} cannot be detected in all of those types of LCs. In addition, the expression of p21, p57 and p18 is not altered by *p27* deletion. (Supplementary data 3). These data suggested that p27 might play a unique role in the pathogenesis of MA9-AML. Consistent with its function, we found that expression levels of p27 are well correlated to the proliferative

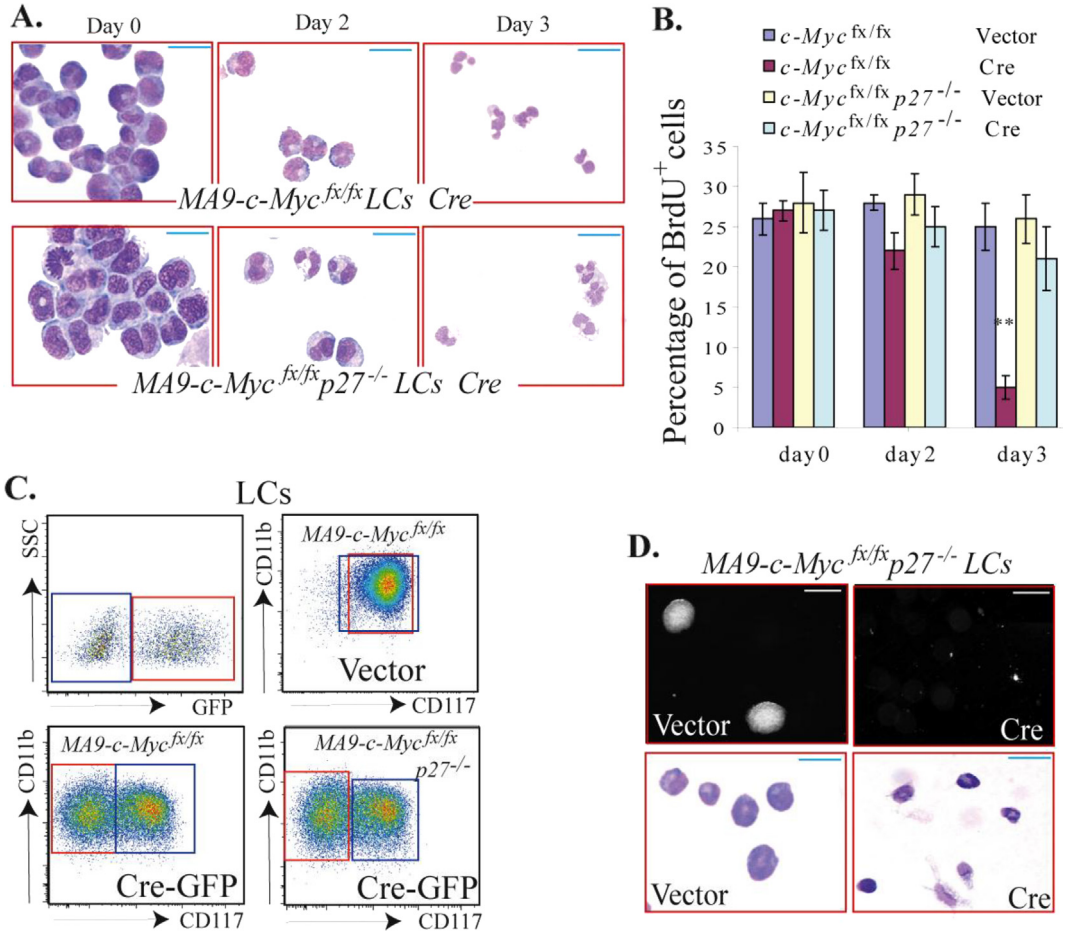

Figure 3 — p27-deficiency prevents cell cycle arrest but not differentiation in MA9-LCs induced by c-Myc deletion. CD117^{+} HSPCs from *c-Myc$^{fx/fx}$* (conditional *c-Myc* knockout) mice and *c-Myc$^{fx/fx}$p27$^{-/-}$* mice were infected with MA9-expressing retrovirus. The infected cells were transplanted into mice for leukemia development. *c-Myc$^{fx/fx}$* MA9-LCs and *c-Myc$^{fx/fx}$p27$^{-/-}$* MA9-LCs were collected from the spleens of leukemic mice and infected with Cre-GFP-expressing retrovirus to induce *c-Myc* deletion. Vector-only virus infection was studied in parallel as a control. A & B. Infected cells were purified by FACS of GFP^{+} cells on the 2nd day after infection and cultured in 4-cytokine medium. Differentiation and proliferation of infected cells were examined on days 0, 2 and 3 after sorting. The differentiation of infected cells was evaluated by morphology (A) and proliferation was examined after 2 h of BrdU pulse-labeling (B). C. The mixture of infected and uninfected cells was cultured in 4-cytokine medium for 4 days. Differentiation of the infected cells (GFP^{+} in red gate) was examined by detecting CD117 expression using flow cytometry. *c-Myc*-deleted cells (Cre-infected) became CD117^{-}CD11b^{+} differentiated cells despite their *p27* status, whereas *c-Myc* intact-LCs (GFP^{-} uninfected cells in blue gate and vector-only control cells) retained their CD117^{+}CD11b^{+} undifferentiated status. D. Infected cells were purified by FACS and seeded into *MethoCult*® GF M3434 medium for 5 days for colony-forming analysis (C, upper panel; photomicrographs were obtained using the same magnification). The morphology of the cells from the colonies was examined by Wright's Giemsa staining (C, lower panel). ** in B indicates significant reduction compared to other groups ($p < 0.01$). Bars represent 50 μm in A and lower panel of D; bar is 0.1 μm in upper panel of D.

and cell cycle states of LCs (Figure 2D and E). In MA9-LCs, the CD117^{+}CD11b^{lo} population is relatively quiescent compared to the CD117^{+}CD11b^{hi} population, as shown by both BrdU pulse-labeling (Figure 2D) and pyronin-Y/Hoechst staining (Supplementary data 4C), whereas in H/M-LCs, proliferation of the CD117^{+}CD11b^{lo} population is comparable to that of the CD117^{+}CD11b^{hi} population (Figure 2E). Proliferation of the CD117^{-}CD11b^{hi} population in both types of LCs is significantly reduced (Figure 2D and E). Furthermore, we found that *p27* deletion significantly promotes the proliferation of the CD117^{+}CD11b^{lo} population of MA9-LCs (Figure 2D) and the CD117^{-}CD11b^{hi} population of both types of LCs (Figure 2D and E), but does not affect the proliferation of the CD117^{+}CD11b^{hi} population in either type of LC (Figure 2D and E). It was known that the CD117^{-}CD11b^{hi} population consists of differentiated LCs whereas LSCs are enriched in CD117^{+} LCs. We found that the CD117^{-}CD11b^{hi} population of both types of LCs contains differentiated granulocytes as shown by Wright's Giemsa staining (Figure 2F and G). The differentiated state of CD117^{-}CD11b^{hi} populations in both types of LCs is not affected by *p27* deletion (Figure 2F and G). These data suggested that p27 inhibits the proliferation of both MA9 and H/M LCs but is not required for the differentiation of either type. The difference in p27 expression and cell cycle state of the CD117^{+}CD11b^{lo} population between MA9-LCs and H/M-LCs indicated that p27 might be required for maintaining the quiescent state in a small subset of MA9-LSCs but not of H/M-LSCs.

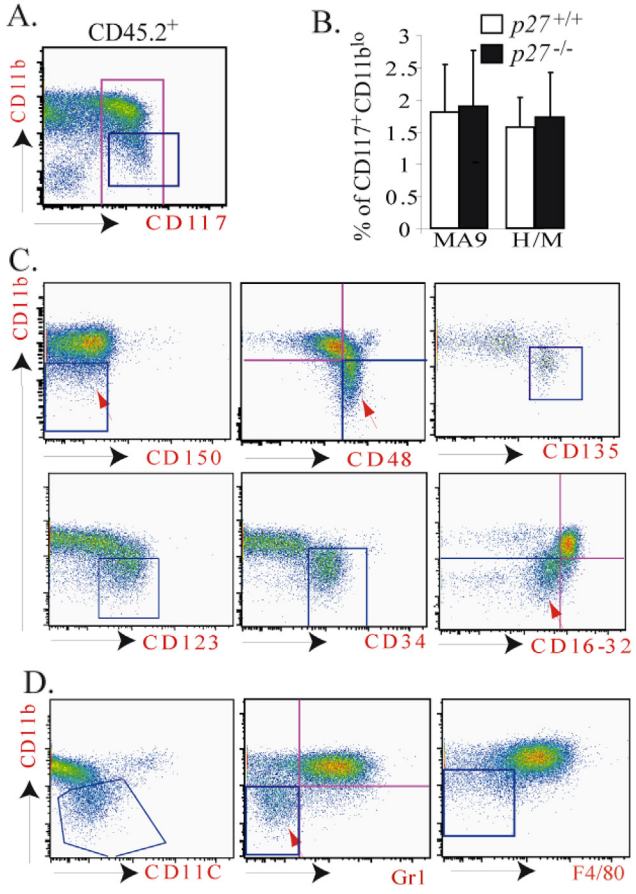

Figure 4 – CD117^{+}CD11b^{lo} LCs express early hematopoietic progenitor markers and lower levels of myeloid cell markers. LCs were collected from the spleens of leukemic mice and stained with cell surface markers as indicated. The phenotypes of LCs were analyzed by flow cytometry. To distinguish LCs from normal hematopoietic cells (both recipient and support BM cells are CD45.1^{+}), cells were first gated on CD45.2^{+} (for MLL-AF9 leukemia) or GFP^{+}/YFP^{+} (for HoxA9/Meis1 leukemia) and then gated on CD117^{+} for further analysis (A). The percentages of CD117^{+}CD11b^{lo} populations in *p27*$^{+/+}$ and *p27*$^{-/-}$ MA9-LCs as well as in *p27*$^{+/+}$ and *p27*$^{-/-}$ H/M-LCs were summarized (B). CD117^{+} LCs (the cells inside the red gate of figure A) were analyzed for expression of HSPC markers (C) and mature myeloid or B lymphocyte markers (D). Cells in the blue gate are CD117^{+}CD11b^{lo} populations in all figures. Data shown in C and D are from *p27*$^{+/+}$ MA9-LCs. Similar data were obtained from *p27*$^{-/-}$ MA9-LCs, *p27*$^{+/+}$ H/M-LCs and *p27*$^{-/-}$ H/M-LCs (data not shown).

3.3. *Proliferation and differentiation of LCs are uncoupled and controlled by p27 and c-Myc respectively*

c-Myc has been shown to play a critical role in the pathogenesis of most acute leukemias by promoting the proliferation and blocking the differentiation of HSPCs (Hoffman et al., 2002; Weng et al., 2006; Fang et al., 2009; Schreiner et al., 2001). We found that c-Myc is expressed in both CD117^{+}CD11b^{lo} and CD117^{+}CD11b^{hi} LC populations but is significantly down-regulated in the CD117^{-}CD11b^{hi} population of both MA9- and H/M-LCs (Figure 2A–B). c-Myc expression correlates well with the differentiation state but not the proliferation rate of LCs (Figure 2). Moreover, we found that knockout of *c-Myc* (*c-Myc*$^{-/-}$) promotes MA9-LC differentiation followed by cell cycle arrest (Figure 3A and B). Inactivation of *p27* can largely prevent cell cycle arrest in LCs without significantly affecting differentiation induced by *c-Myc* gene deletion as shown in Figure 3A–C. In colony-forming assays, we found that *c-Myc*$^{-/-}$ LCs can only divide 1–3 times and form 2- to 8-cell clusters (**data not shown**), whereas many of the *c-Myc*$^{-/-}$*p27*$^{-/-}$ LCs were able to divide for 4–5 times and generate clusters of 10–30 cells. Cells from these clusters are mature granulocytes (Figure 3D). However, despite the fact that they were proliferating, due to their complete maturation after day 3 of culturing (Figure 3A), *c-Myc*$^{-/-}$*p27*$^{-/-}$ LCs fail to form large colonies.

3.4. CD117^{+}CD11b^{lo} *populations of MA9-LCs and H/M-LCs express early hematopoietic progenitor markers regardless of their* p27 *status*

We found that populations of both MA9-LCs and H/M-LCs consist of 1–3% of CD117^{+}CD11b^{lo} cells (Figure 4A and B). This percentage in both types of LCs is not affected by *p27* deletion (Figure 4B). Morphologically, for both types of LCs, the CD117^{+}CD11b^{lo} cells are relatively smaller with higher nucleus-to-cytoplasm ratios compared to those in the CD117^{+}CD11b^{hi} population. Again, this type of morphologic distinction is not altered by *p27* deletion (Figure 2F and G).

To study whether the CD117^{+}CD11b^{lo} population of LCs is phenotypically different from the CD117^{+}CD11b^{hi} population,

we compared the expression of hematopoietic cell markers between these two populations in both MA9-LCs and H/M-LCs. We found that in both types of LCs, compared to the CD117^{+}CD11b^{hi} population, the CD117^{+}CD11b^{lo} populations express higher levels of several HSPC markers, including CD48, CD123 (IL3-receptor), and CD135 (Flt3) (Figure 4C) but lower levels of mature myeloid cell markers, including Gr1, CD11C, F4/80 and CD16/32 (Figure 4D). In addition, in contrast to the CD117^{+}CD11b^{hi} population, which represents a mixture of CD34^{+} and CD34^{-} cells (Supplementary data 4A), almost all CD117^{+}CD11b^{lo} LCs are CD34^{+} (Figure 4C and Supplementary data 4A). Within the CD117^{+}CD11b^{hi} population, CD34^{+} and CD34^{-} cells behaved similarly in terms of their proliferation and colony-forming ability (Supplementary data 4C and D). These data indicated that CD117^{+}CD11b^{lo} LCs might be relatively undifferentiated compared to CD117^{+}CD11b^{hi} LCs.

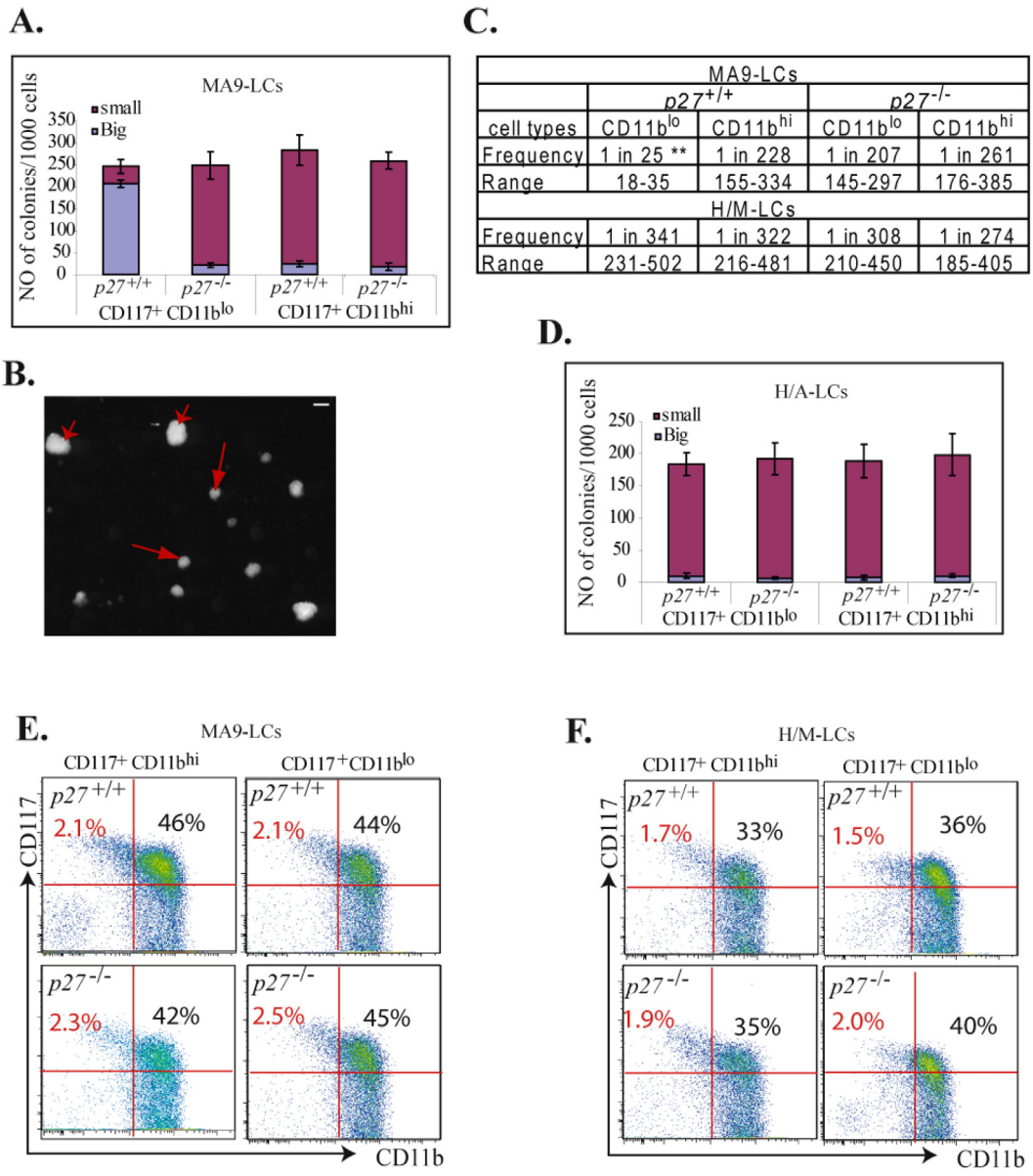

Figure 5 – CD117^{+}CD11b^{lo} MA9-LCs contain a higher frequency of leukemogenic LSCs than do CD117^{+}CD11b^{hi} MA9-LCs. A-D. LCs were collected from the spleens of *p27*$^{+/+}$ MA9 (CD45.2^{+}) and *p27*$^{+/+}$ H/M (GFP^{+}/YFP^{+}) leukemic mice and were separated into CD117^{+}CD11b^{lo}, CD117^{+}CD11b^{hi} and CD117^{-}CD11b^{hi} populations by FACS. Cells were seeded into *MethoCult*® GF M3434 medium for colony-forming analysis. Clonogenic ability of CD117^{+}CD11b^{lo}, CD117^{+}CD11b^{hi} and CD117^{-}CD11b^{hi} populations was compared between *p27*$^{+/+}$ MA9-LCs and *p27*$^{-/-}$ MA9-LCs (A), as well as between *p27*$^{+/+}$ H/M-LCs and *p27*$^{-/-}$ H/M-LCs (D). Large and small colonies were counted in each sample. The size of large colonies (arrowhead) is 2–3 times larger than the size of small colonies (arrow) as shown in B. The frequency of LSCs in the CD117^{+}CD11b^{lo} and CD117^{+}CD11b^{hi} populations in *p27*$^{+/+}$ MA9-LCs, *p27*$^{-/-}$ MA9-LCs, *p27*$^{+/+}$ H/M-LCs and *p27*$^{-/-}$ H/M-LCs was examined by serial dilution and competitive repopulation assays (C). ** indicates significant increase compared to other groups ($p < 0.01$). The percentages of CD117^{+}CD11b^{lo} and CD117^{+}CD11b^{hi} populations of cells were examined in leukemic mice which had received CD117^{+}CD11b^{lo} and CD117^{+}CD11b^{hi} LCs (E & F). Bar in B represents 0.1 μm.

3.5. CD117$^+$CD11b^{lo} MA9-LCs contain a higher frequency of leukemogenic LSCs than CD117$^+$CD11b^{hi} MA9-LCs, which can be normalized by p27 deletion

To study whether the CD117$^+$CD11b^{lo} population of LCs is functionally different from the CD117$^+$CD11b^{hi} population, we compared the *in vitro* colony-forming ability and *in vivo* leukemogenic capacity between these two populations in both MA9-LCs and H/M-LCs. We found that CD117$^+$CD11b^{lo} MA9-LCs generated significantly more large colonies (2–3 times more cells in each colony) (Figure 5A, B and Supplementary data 4D) and contained a significantly higher frequency of leukemogenic LSCs than did CD117$^+$CD11b^{hi} MA9-LCs (Figure 5C and Supplementary data 5). Interestingly, *p27* deletion significantly compromised the leukemogenic capacity of the CD117$^+$CD11b^{lo} population, which makes the CD117$^+$CD11b^{lo} population equivalent to the CD117$^+$CD11b^{hi} population in terms of their *in vitro* colony growth and *in vivo* leukemogenic capacity (Figure 5A and C). However, CD117$^+$CD11b^{lo} H/M-LCs generated comparable numbers/sizes of colonies and contained a comparable frequency of leukemogenic LSCs as CD117$^+$CD11b^{hi} H/M-LCs. The clonogenic ability and leukemogenic capacity of H/M-LCs are independent of their *p27* status (Figure 5C and D). Furthermore, mice receiving CD117$^+$CD11b^{lo} and CD117$^+$CD11b^{hi} LCs developed AML of exactly the same phenotype as the donor mice from which the cells had been harvested, being composed of similar percentages of CD117$^+$CD11b^{lo} and CD117$^+$CD11b^{hi} cells (Figure 5E and F). These data suggested that p27 is required to maintain the leukemogenic capacity of CD117$^+$CD11b^{lo} MA9-LCs but not H/M-LCs. The CD117$^+$CD11b^{lo} and CD117$^+$CD11b^{hi} populations are, in effect, phenotypically inter-convertible *in vivo*, with each population being able to give rise to the other.

3.6. p27 can be induced by Flt3-ligand and SCF, and its expression is required to maintain drug resistance in CD117$^+$CD11b^{lo} MA9-LCs

To study whether *p27* expression in MA9-LCs can be stimulated by certain cytokines, we isolated *p27*$^{+/+}$ CD117$^+$ MA9-LCs from the spleens of leukemic mice and treated them with the indicated concentration of cytokines. *p27*$^{+/+}$ CD117$^+$ H/M-LCs were studied in parallel as controls. Interestingly, we found that, in MA9-LCs, *p27* expression was significantly induced by Flt3 Ligand (Flt3L) and SCF stimulation but not by IL-3 nor GM-CSF, as shown by both Western Blotting (Figure 6A) and qRT-PCR (Figure 6B). In contrast, in H/M-LCs, none of these cytokines increased *p27* expression.

Because the CD117$^+$CD11b^{lo} population of MA9-LCs is relatively quiescent compared to the CD117$^+$CD11b^{hi} population, we predicted that the former cells might be more resistant to chemotherapeutic drug treatment. To test this hypothesis, we treated CD117$^+$CD11b^{lo} and CD117$^+$CD11b^{hi} LCs with different dosages of cytosine arabinoside (Ara-C) or daunorubicin (DNR) for 6 h. The drug sensitivity of the cells was evaluated by both Annexin-V staining and colony-forming assay. We found that the CD117$^+$CD11b^{lo} population of MA9-LCs is relatively more resistant to both Ara-C and DNR treatment compared to the CD117$^+$CD11b^{hi} population (Figure 7A and C). We believe that this drug resistance in the CD117$^+$CD11b^{lo} population of MA9-LCs is p27-dependent, as the cells can be re-sensitized to the drugs' effects by *p27* deletion (Figure 7A and C). However, the sensitivity of CD117$^+$CD11b^{lo} H/M-LCs to Ara-C is comparable to that of the CD117$^+$CD11b^{hi} population (Figure 7B and D).

3.7. Forced p27 expression inhibits proliferation and induces drug resistance in the CD117$^+$CD11b^{lo} population of H/M-LCs

To further investigate whether the functional difference of the CD117$^+$CD11b^{lo} population in MA9-LCs compared to H/M-LCs is due to p27, we forced the expression of p27 in the CD117$^+$CD11b^{lo} population isolated from H/M-LCs. We found that p27 expression inhibits proliferation in such cells as shown by BrdU pulse-labeling (Figure 8A). We also found that p27-transduced CD117$^+$CD11b^{lo} H/M-LCs are relatively more resistant to chemotherapeutic drug treatment than vector-only-transduced CD117$^+$CD11b^{lo} H/M-LCs (Figure 8B and C). These data further support the key role of p27 in inducing the quiescent/drug-resistant feature of CD117$^+$CD11b^{lo} LCs.

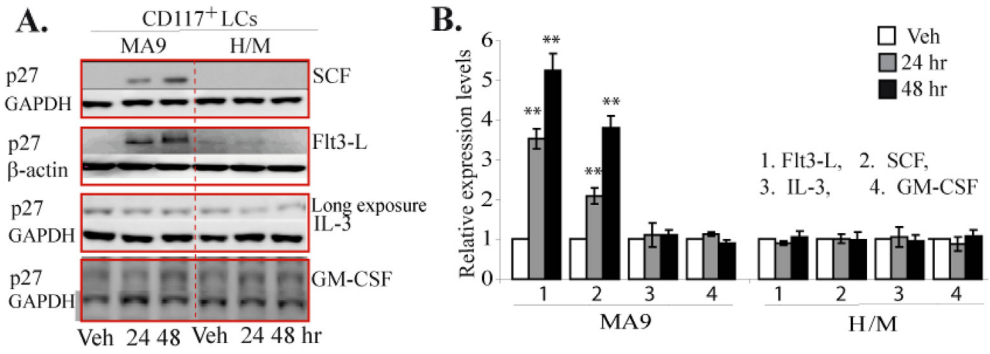

Figure 6 – p27 expression can be induced by Flt3 and SCF in CD117$^+$ MA9-LCs but not in CD117$^+$H/M-LCs. CD117$^+$ LCs were isolated from spleens of MA9 and H/M leukemic mice by FACS and incubated in RPMI 1640 medium supplemented with 10% FBS. Cells were treated with 100 ng/ml Flt3, 100 ng/ml SCF, 20 ng/ml IL-3 or 25 ng/ml GM-CSF for the indicated times. p27 expression was examined by Western Blotting (A) and qRT-PCR (B). Vehicle (Veh) treated cells were used as controls. In B, the relative levels of mRNA expression were first normalized to the expression levels of β-actin in each sample and then further normalized to the levels in control samples of each group. ** indicates significant increase compared to other groups ($p < 0.01$).

4. Discussion

LSCs have been implicated as central to the processes involved in the progression and relapse of leukemia. The complete elimination of all LSCs from patients' tissues, therefore, has been proposed to be the ultimate and necessary goal in curing this fatal disease (Konopleva and Jordan, 2011; Becker and Jordan, 2011). However, accumulated evidence suggests that LSCs representing a special subset of LCs are able to survive intensive chemotherapeutic interventions because of the influence of niche microenvironmental factors (Saito et al., 2010a,b). Maintaining quiescence has been proposed to be one of the mechanisms by which LSCs evade the cytotoxic effects of chemotherapeutic agents because most currently-available chemotherapeutic drugs used to treat leukemia typically target cycling cells (Weissman, 2005; Dick, 2005; Jamieson et al., 2004; Reya et al., 2001; Guzman and Jordan, 2004). In-depth studies of the molecular mechanisms by which LSCs maintain their state of replicative quiescence should help point the way toward the development of novel strategies to induce such cells into the cell cycle and hence become susceptible to chemotherapeutic killing. We found that, in spite of the phenotypic similarity between MA9-LCs and H/M-LCs, within the population of $CD117^+$ undifferentiated LCs, p27 is specifically expressed in only a small subset of $CD11b^{lo}$ MA9-LCs but not in the corresponding population in H/M-LCs. $CD117^+CD11b^{lo}$ MA9-LCs are relatively quiescent and consist of a greater percentage of LSCs than other populations. *p27* expression can be induced by Flt3-L and SCF in MA9-LCs, which is required for maintaining the quiescent state, drug resistance and leukemogenic capacity of $CD117^+CD11b^{lo}$ MA9-LCs. We propose that Flt3-L and SCF might stimulate niche signals to specifically protect these populations of LSCs from cytotoxic stress by inducing *p27* expression and restricting their proliferation. This model might explain the poor prognosis associated with MLL-fusion gene-related leukemias compared to non-MLL leukemias (He et al., 2011; Krivtsov and Armstrong, 2007) and in general the more favorable prognosis of leukemias with low levels of p27 expression compared to leukemias in which p27 is expressed at high levels (Haferlach et al., 2011). The molecular mechanism by which Flt3-L and SCF specifically induce p27 in MA9-LCs but not H/M-LCs need to be addressed. In addition, clinical studies have suggested that a high level HoxA9 is also key marker of poor prognosis in leukemia (Li et al., 2013). Future studies need to determine whether the prognosis of leukemia with high levels of both p27 and HoxA9 is even poorer than leukemia with high levels of either p27 or HoxA9.

p27 is a member of the cip/kip cyclin-dependent kinase inhibitor (CDKi) family of proteins which suppress cell cycle progression by repressing the activities of cyclinE-CDK2 complexes in late G_1 phase and cyclin A-CDK2 in early S phase. Members of this family of proteins have been shown

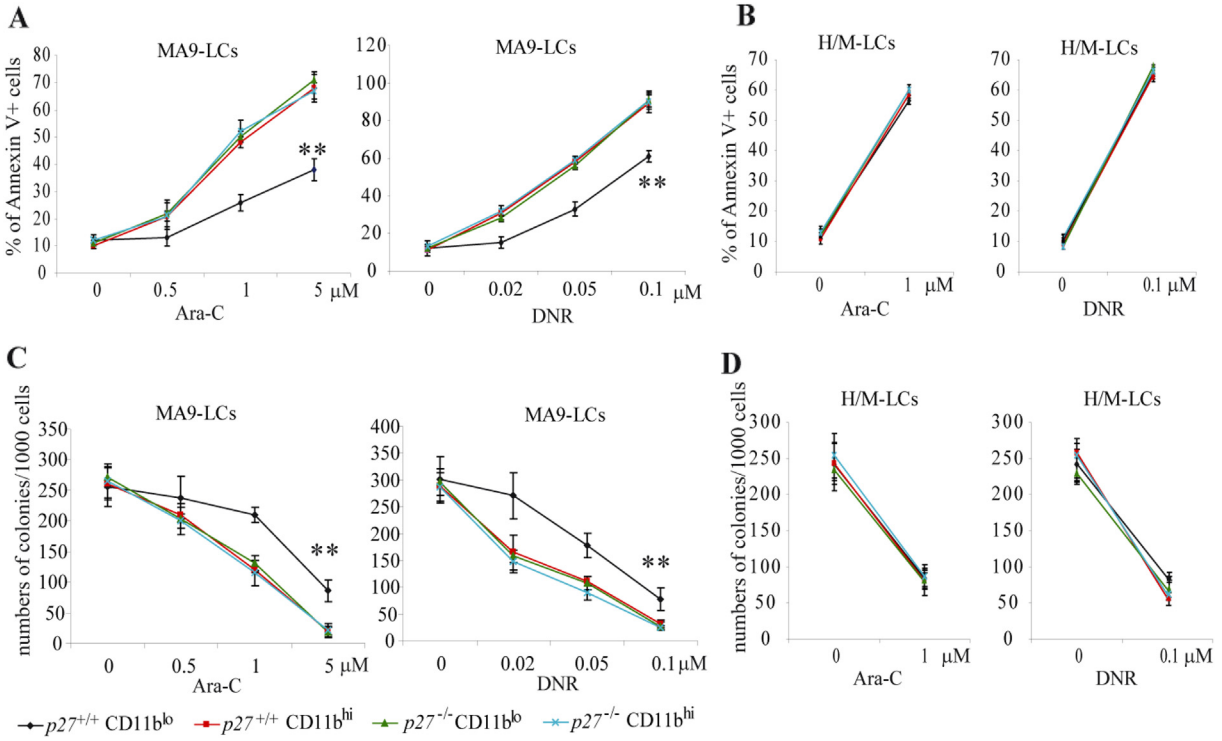

Figure 7 – $CD117^+CD11b^{lo}$ LCs are relatively more drug resistant than are $CD117^+CD11b^{hi}$ LCs. Populations of $CD117^+CD11b^{lo}$ and $CD117^+CD11b^{hi}$ LCs were isolated from the spleens of $p27^{+/+}$ MA9, $p27^{-/-}$ MA9, $p27^{+/+}$ H/M and $p27^{-/-}$ H/M leukemic mice by FACS. Cells were incubated in 96-well plates in 4-cytokine culture medium with indicated dosages of cytosine arabinoside (Ara-C) or doxorubicin (DNR). Cells were seeded at a density of 2×10^4 or 1×10^3 per well for cell death or colony-forming analysis, respectively. Cell death was determined after 16 h of treatment by Annexin-V staining and analyzed by flow-cytometry (A & B). The remaining colony-forming cells in each well after 6 h of Ara-C or doxorubicin treatment were examined by suspension culture in *MethoCult*® GF M3434 (C & D). ** indicates significant difference compared to other groups ($p < 0.01$).

to play critical roles in normal hematopoiesis by restricting the proliferation of both HSCs and hematopoietic progenitors. p57 is highly expressed in HSCs and is down-regulated during differentiation (Umemoto et al., 2005). It has been demonstrated that p57 expression can be induced by BM niche signals such as TPO and TGF1β (Yamazaki et al., 2006; Yamazaki et al., 2009; Yoshihara et al., 2007; Scandura et al., 2004), which are required to maintain the quiescent state and self-renewal capacity of primitive long-term HSCs (Matsumoto et al., 2011; Zou et al., 2011). The expression of p21 is low in HSCs and is increased in multipotent progenitors (MPPs). Studies have demonstrated that p21 is a key effector of p53-mediated G_1 cell cycle arrest and apoptosis, whose expression can be induced by treatment with various genotoxic agents or by oncogene-induced DNA damage (Seoane et al., 2002; Viale et al., 2009). p21 deficiency has fewer effects on normal hematopoiesis during homeostasis but sensitizes HSCs to DNA damage-induced depletion stimulated by myelotoxic injury (Cheng et al., 2000a,b; van Os et al., 2007). p27 is consistently expressed in HSCs and MPPs at a relatively low level. Due to the compensation of p57, p27 deficiency has no effect on the self-renewal and proliferation of HSCs but significantly induces the expansion of MPPs (Cheng et al., 2000a,b). Interestingly, despite evidence of their growth inhibitory function on LCs, as shown by *in vitro* culture studies, there have not yet been any reports of mutations within this family of genes in human leukemias. The role of this family of CDKi's in the pathogenesis of leukemia remains elusive.

Leukemia cell line studies have indicated that *p27* expression is normally repressed in undifferentiated LCs and is markedly up-regulated during differentiation, which is commonly correlated with *c-Myc* down-regulation. *p27* over-expression induces LC differentiation and cell cycle arrest (Munoz-Alonso et al., 2005). These studies suggested that differentiation is always coupled with arrest of the cell cycle. However, in our current studies, we found that in both MA9-LCs and H/M-LCs, *p27* deletion promotes the proliferation of $CD117^-$ differentiated LCs without affecting their differentiation state, as shown by cell marker staining, morphology and colony-formation assays. Our studies indicate that differentiation and proliferation of LCs are controlled by c-Myc and p27 respectively, which is consistent with a previous report. In this report, Acosta et al. found that *c-Myc* over-expression can prevent differentiation but fails to rescue the inhibition of proliferation of K652 cells induced by p27 over-expression (Acosta et al., 2008). Interestingly, in $CD117^+CD11b^{lo}$ MA9-LCs, which are enriched in quiescent LSCs, both *c-Myc* and *p27* are expressed. We predict that c-Myc prevents differentiation and collaborates with p27 to maintain LSCs in quiescence in murine MA9-leukemia. Whether this co-expression of c-Myc and p27 is also required to maintain the quiescence and drug resistance of LSCs in human MLL-leukemias needs additional study.

p27 has both tumor suppressive and tumor promoting activities, thus playing a dual role in tumorigenesis (Besson et al., 2007). The tumor suppressive activity of p27 is accomplished in the nucleus through inhibition of CDK-dependent cell cycle progression, whereas its tumor promoting activity is cytoplasmic, through the regulation of RhoA activation or possibly a currently-unknown mechanism which is CDK-independent (Besson et al., 2004). We speculate that the role

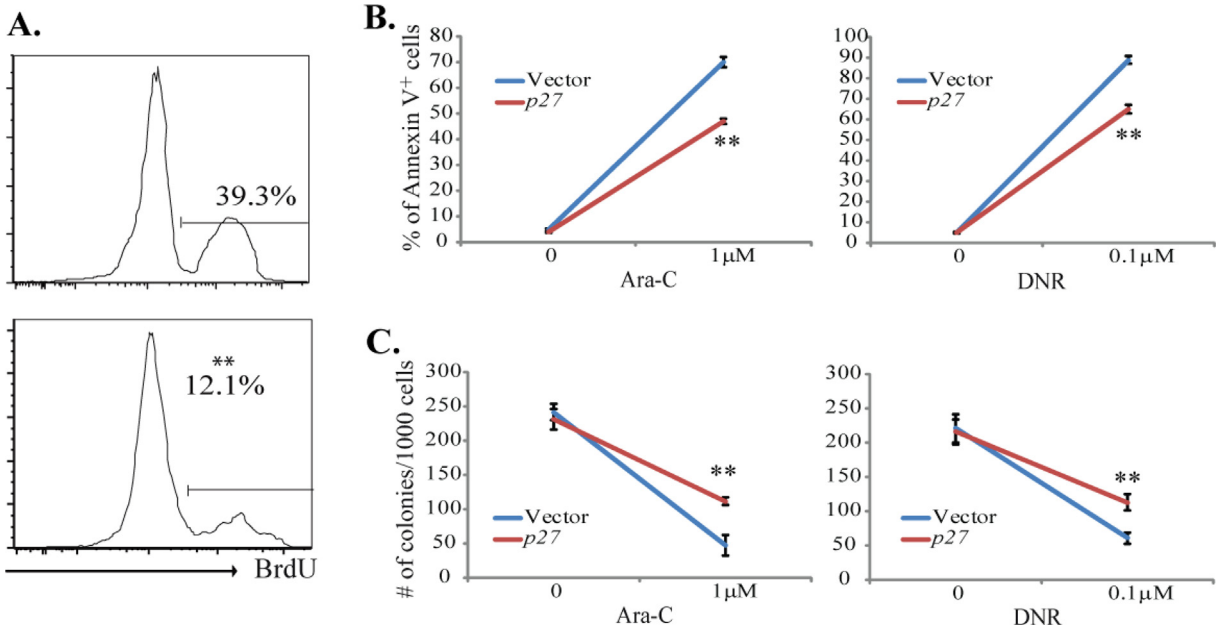

Figure 8 — p27 over-expression represses proliferation and induces drug resistance in $CD117^+CD11b^{lo}$ H/M-LCs. $CD117^+CD11b^{lo}$ cells were sorted from H/M-LCs and infected with p27-mcherry-expressing virus. Infected cells were purified one day after infection by sorting $mcherry^+$ cells and were incubated in 4-growth factor medium for 24 h mCherry vector-only infected cells were studied in parallel as a control. A. Cells were treated with 10 ng/ml BrdU 2 h prior to collection. Cell proliferation was examined by detecting the $BrdU^+$ cell percentage. B. Cells were seeded at a density of 2×10^4 per well and treated with indicated dosages of Ara-C or DNR. Cell death was determined 16 h after the initiation of treatment by Annexin-V staining and analyzed by flow-cytometry. C. Cells were seeded at a density of 1×10^3 per well and treated with indicated dosages of Ara-C or DNR. The remaining colony-forming cells in each well after 6 h of Ara-C or DNR treatment were examined by suspension culture in *MethoCult*® GF M3434. **indicates significant difference compared to other groups ($p < 0.01$).

p27 plays in murine MA9 and H/M leukemias is accomplished through a CDK-dependent mechanism because deletion of the *p27* gene mainly alters the proliferation of both types of LCs while having minor effects on the leukemogenic ability of MA9-LCs (only within the $CD117^{+}CD11b^{lo}$ population). Whether this CDK-independent pro-oncogenic function of p27 is involved in the pathogenesis of other types of leukemia needs to be addressed.

Quiescence is one of the key features of self-renewable HSCs (Wilson et al., 2008). It was demonstrated that long-term reconstitutive HSCs are largely enriched in quiescent cell populations. Inducing cell cycle entry of HSCs will significantly compromise their function (Baldridge et al., 2010). This quiescent state of HSCs is essentially maintained by p57, with additional support from p27 (Matsumoto et al., 2011). Studies of clinical samples have indicated that LSCs are also quiescent (Saito et al., 2010a; Zou et al., 2011). A recent study suggested that in murine PML-RARα and AML1–ETO leukemia models, p21 is indispensable for maintaining self-renewal in LSCs by restricting cellular proliferation and limiting the accumulation of DNA damage. LSCs with p21 deficiency will be exhausted through apoptosis and senescence as a consequence of the marked accumulation of DNA damage (Viale et al., 2009). Whether p21 is required for LSC self-renewal in MLL-related leukemias has not yet been studied. We found that LSCs are enriched in both $CD117^{+}CD11b^{hi}$ and $CD117^{+}CD11b^{lo}$ populations of MA9-LCs. *p27* deletion affects only the proliferation and leukemogenic ability of $CD117^{+}CD11b^{lo}$ LSCs, having no influence on the $CD117^{+}CD11b^{hi}$ population. We are currently in the process of investigating whether LSCs in the $CD117^{+}CD11b^{hi}$ population are also quiescent. If so, we will subsequently determine which CDKi, p21 or p57, restricts the proliferation of these LSCs, and also which niche signal(s) induce(s) cell cycle arrest in these LSCs.

Contributions

Jun Zhang, Christopher Seet, Clear Sun, Jing Li, Dewen You, Yechen Xiao, Andrew Volk, Peter Breslin, Shubin Zhang and Wei Wei performed the research and analyzed the data. Nancy J. Zeleznik-Le, Manual Diaz, Zhijiang Qian and Jiwang Zhang designed the experiments. Peter Breslin, Zhou Zhang and Jiwang Zhang analyzed the data and wrote the paper.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Acknowledgments

We appreciate the excellent animal care services provided by the staff of the Department of Comparative Medicine, Loyola University Medical Center. This work was supported by NIH – R01 HL95896, NSFC Project 81071774, 31201104 and 31270883, National Basic Research Program of China 2013CB966800, the Program for Professor of Special Appointment (Eastern Scholar) at the Shanghai Institutions of Higher Learning, the Food Safety and Nutrition Program of Shanghai Normal University DXL123, the Innovation Program of Shanghai Municipal Education Commission 13ZZ103 and 13YZ051, as well as by a grant from the Jimmy Burns Foundation.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2013.07.011.

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