CD93 Marks a Non-Quiescent Human Leukemia Stem Cell Population and Is Required for Development of MLL-Rearranged Acute Myeloid Leukemia

Graphical Abstract

Highlights
- Cell surface lectin CD93 is a functional marker of LSCs in MLL-rearranged AML
- CD93+ LSCs are cycling, non-quiescent leukemia-initiating cells
- LSC expression of CD93 is essential for MLL-mediated leukemogenesis
- CD93 regulates LSC self-renewal by silencing CDKN2B in MLL leukemia

Authors
Masayuki Iwasaki, Michaela Liedtke, Andrew J. Gentles, Michael L. Cleary

Correspondence
mcleary@stanford.edu

In Brief
Iwasaki et al. demonstrate that leukemia stem cells (LSCs) in a distinctive genetic subtype of leukemia are non-quiescent. Although human LSCs are typically enriched in the highly quiescent CD34+CD38− phenotypic compartment, co-expression of the lectin CD93 further demarcates LSCs as a discrete subpopulation of actively cycling, non-quiescent AML cells.

Accession Numbers
GSE64773
GSE64776

Iwasaki et al., 2015, Cell Stem Cell 17, 412–421
October 1, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.stem.2015.08.008
CD93 Marks a Non-Quiescent Human Leukemia Stem Cell Population and Is Required for Development of MLL-Rearranged Acute Myeloid Leukemia

Masayuki Iwasaki,1 Michaela Liedtke,2 Andrew J. Gentles,3 and Michael L. Cleary1,*
1Department of Pathology
2Department of Medicine
3Department of Radiology
Stanford University School of Medicine, Stanford, CA 94305, USA
*Correspondence: mcleary@stanford.edu
http://dx.doi.org/10.1016/j.stem.2015.08.008

SUMMARY

Leukemia stem cells (LSCs) are thought to share several properties with hematopoietic stem cells (HSCs), including cell-cycle quiescence and a capacity for self-renewal. These features are hypothesized to underlie leukemic initiation, progression, and relapse, and they also complicate efforts to eradicate leukemia through therapeutic targeting of LSCs without adverse effects on HSCs. Here, we show that acute myeloid leukemias (AMLs) with genomic rearrangements of the MLL gene contain a non-quiescent LSC population. Although human CD34+CD38− LSCs are generally highly quiescent, the C-type lectin CD93 is expressed on a subset of actively cycling, non-quiescent AML cells enriched for LSC activity. CD93 expression is functionally required for engraftment of primary human AML LSCs and leukemogenesis, and it regulates LSC self-renewal predominantly by silencing CDKN2B, a major tumor suppressor in AML. Thus, CD93 expression identifies a predominantly cycling, non-quiescent leukemia-initiating cell population in MLL-rearranged AML, providing opportunities for selective targeting and eradication of LSCs.

INTRODUCTION

Acute myeloid leukemia (AML) is organized as a cellular hierarchy, relying on self-renewing leukemia stem cells (LSCs) at the apex to generate non-self-renewing blasts and differentiated progeny cells. LSCs typically comprise a small minority of cells within the hierarchy, but their relative abundance may be clinically significant since recent studies suggest that detection of an LSC or “stemness” gene expression signature in AML is associated with a poor prognosis (Gentles et al., 2010; Eppert et al., 2011). From a clinical perspective, it has been demonstrated that LSCs are resistant to standard chemotherapy and are retained and give rise to relapse (Jordan et al., 2006; Ishikawa et al., 2007; Konopleva and Jordan, 2011). Thus, LSCs are the ultimate therapeutic target to eradicate AML without killing normal hematopoietic stem cells (HSCs), but this strategy depends on identifying and exploiting biomarkers or pathways that are differentially expressed by LSCs and may functionally sustain leukemogenesis.

Both HSCs and LSCs share the properties of self-renewal, quiescence, and expression of a CD34+CD38− immunophenotype (Bhatia et al., 1997; Bonnet and Dick, 1997; Lapidot et al., 1994). However, in some cases LSCs may be phenotypically distinguished from HSCs by preferential expression of cell surface proteins such as CD123, CD44, CLL-1, CD96, CD47, CD25, CD32, or TIM-3 (Jordan et al., 2000; Jin et al., 2006; van Rhenen et al., 2007; Hosen et al., 2007; Jaiswal et al., 2009; Majeti et al., 2009; Saito et al., 2010; Kikushige et al., 2010; Jan et al., 2011). Expression of TIM-3 is particularly discriminatory, capable of distinguishing highly mutated LSCs from less mutated pre-leukemic HSCs (Jan et al., 2011), suggesting a potential function underling the progressive evolution of mutated HSCs to bona fide LSCs. Other differentially expressed antigens function in immune recognition or its evasion, thus providing a mechanistic rationale as candidate targets for immunotherapies (Jin et al., 2006, 2009; Majeti et al., 2009; Kikushige et al., 2010). However, not all AML subtypes consistently express the foregoing antigens, raising the issue of whether alternative surface markers may be selectively expressed by LSCs in specific genetic subtypes of AML. Furthermore, surface markers that distinguish LSCs versus HSCs have not generally been mechanistically linked with essential LSC functions, particularly those that underlie self-renewal or cell-cycle regulation. Although LSCs are enriched in a highly quiescent phenotypic compartment of the AML hierarchy, they must nevertheless actively cycle to self-renew, sustain disease, and facilitate clinical relapse. It is likely that LSC phenotypes and cycling characteristics vary with different genetic subtypes of AML, which are remarkably diverse in terms of developmental and pathological characteristics.

Here we report that the cell surface lectin CD93 is a functional marker of LSCs in a specific genetic subtype of AML with genomic rearrangements of the MLL (Mixed Lineage Leukemia) gene. CD93 selectively marks and essentially maintains LSCs and identifies them as predominantly cycling, non-quiescent leukemia-initiating cells. CD93 promotes LSC proliferation in part through silencing of CDKN2B, a major cell-cycle inhibitor and tumor suppressor whose expression is silenced in a majority of AMLs with poor prognosis.
RESULTS

Identification of Surface Markers Differentially Expressed by LSCs

A leukemia cell fraction highly enriched for LSCs was generated in a mouse model of AML induced by co-expression of MLL target genes HoxA9 and Meis1. Limiting dilution transplantation analyses performed on various prospectively isolated leukemia cell subpopulations revealed that cells capable of transplanting AML into syngeneic recipient mice (the operational definition of LSCs) were highly enriched in the leukemia cell fraction, displaying an immunophenotype (Lin- CD34+CD38- CD16/32+CD34+) comparable to that of normal granulocyte macrophage progenitors (GMPs) (Figures S1A and S1B), referred to as L-GMPs.

Gene expression profiling identified 2,291 genes that were differentially expressed (at least 1.5-fold) in L-GMPs versus GMPs (1,027 upregulated versus 1,264 downregulated). Ten of the significantly upregulated genes encoded candidate cell surface molecules (Figure S1C). These included CD93, a C-type lectin implicated in cellular adhesion, whose surface expression was confirmed by flow cytometry (Figure 1A and B) and dot plot summarizing the relative CD93 expression on CD34+CD38- cells from the indicated sources (Figure 1D).

Figure 1. CD93 Expression on Human CD34+CD38- AML and Cord Blood Cells

(A and B) Flow cytometry plots show CD93 expression on CD34+CD38- AML cells of MLLr patient #2 (A) and non-MLLr patient #19 (B).

(C) Flow cytometry plot shows CD93 expression on Lin- CD34+CD38- cord blood cells.

(D) Dot plot summarizes the relative CD93 expression on CD34+CD38- cells from the indicated sources.

See also Figures S1 and S2 and Table S1.

Figure 2. CD34+CD38-CD93+ Human AML Cells Are Highly Enriched in LSC Activity

(A) Bar graph shows CFC frequencies for FACS-sorted CD34+CD38-CD93+ or CD34+CD38-CD93- cells from primary MLLr AMLs (n = 5) plated in methylcellulose for 12 days. Data are the mean number of colonies (±SEM) per 5,000 plated AML cells. *p = 0.0006.

(B) Representative colony morphologies are shown for the experiment in (A).

(C) LSC frequencies were determined by limiting-dilution transplantation of FACS-sorted CD34+CD38-CD93+ or CD34+CD38-CD93- AML subpopulations.

See also Figures S3 and S4 and Tables S2 and S3.
appeared strongly correlated with Meis1 levels in co-expression experiments with either HoxA9 or MLL oncogenes (Figure S2). Thus, we focused our subsequent studies on CD93 as a potential cell surface antigen to functionally delineate LSCs in Hox-associated leukemia containing MLL rearrangements.

CD93 Is a Functional Marker of LSCs in MLL-Rearranged AML

In most human AMLs, LSCs are enriched in the CD34+CD38- cell fraction (Bonnet and Dick, 1997), which also contains normal HSCs and multipotent progenitors. Therefore, we evaluated the CD93 expression profile on CD34+CD38- cells comprising various human AMLs (Table S1) and normal cord blood by flow cytometry (representative results are shown in Figures 1A–1C). CD93 was expressed on a significant, albeit variable, percentage of cells in the CD34+CD38- fraction of MLL-rearranged (MLLr) leukemias (Figure 1D). In contrast, the comparable subpopulations within non-MLLr leukemias or cord blood cells (Lin-CD34+CD38- fraction) generally lacked significant expression of CD93 (Figure 1D) although the bulk AML population in some non-MLLr leukemias contained cells with high CD93 expression. Thus, CD93 is selectively expressed on a subset of cells within the stem/progenitor cell-enriched subpopulation of MLLr AML.

Since CD93 expression phenotypically subdivides the CD34+CD38- population of human MLL leukemia, we assessed whether its presence may correlate with functional properties of LSCs. Colony-forming assays performed on prospectively isolated MLLr AML cells revealed that clonogenic activity was at least 10-fold higher in the CD93+ fraction compared with the CD93- fraction of CD34+CD38- cells (Figure 2A and Figure S3A). The compact morphology of many colonies generated by CD34+CD38-CD93+ cells versus uniformly diffuse colonies induced by CD34+CD38-CD93- cells (Figure 2B) suggested that the former may be composed of a high proportion of proliferating cells. CD93+ also enriched for CFC (colony-forming cell) activity within the CD34+CD38- fraction albeit to a lesser extent (Figure S3A). Very high CD93 levels (CD93++) were present on terminally differentiated non-clonogenic myeloid cells in the un-fractionated AML population (Figure S3B). To determine whether LSCs were more enriched in the CD93+ cell fraction, limiting dilution xenotransplantation experiments were performed in NSG recipient mice. Consistent with the CFC assays, prospectively isolated CD34+CD38-CD93+ cells induced leukemia much more efficiently than CD34+CD38-CD93- cells (Table S2). Estimation of LSC frequencies based on Poisson statistical analysis indicated that LSCs were at least 100-fold more prevalent in the CD34+CD38-CD93+ subpopulation (Figure 2C). CD93 also enriched for LSCs outside of the CD34+CD38- subpopulation (CD34+CD38+) of AML (Table S3). The xenograft leukemia cells were of human origin (hCD45+), displayed myeloid immunophenotypes, and consisted of CD34/CD38/CD93 subpopulations similar to the primary human AMLs (Figure S4). Leukemias serially engrafted secondary transplant recipients (data not shown), indicating that the originally injected CD34+CD38-CD93+ cells were composed of self-renewing LSCs. Taken together, these results indicate that CD93 expression further enriches for a subpopulation of MLLr AML cells with functional properties of LSCs.

CD93+ LSCs in MLL-Rearranged AML Are Non-Quiescent

Global gene expression profiling analysis was performed to identify the genes and pathways that correlated with CD93 expression in MLLr LSCs. Comparison of gene expression profiles of the LSC-enriched (CD34+CD38-CD93+) versus LSC-depleted (CD34+CD38-CD93-) populations from within individual leukemias revealed 374 differentially expressed (at least 1.3-fold) probe sets (258 upregulated versus 116 downregulated). Classification based on gene ontology (GO) showed that the ten most significant annotation groups for upregulated genes in CD34+CD38-CD93+ cells consisted of cell-cycle genes (Figure 3A). Furthermore, gene set enrichment analysis (GSEA) revealed significant enrichment...
for cell-cycle-related gene sets, including Rb- and E2F-regulated genes (Figure 3B). Thus, the CD93+ LSC-enriched subpopulation of MLLr AML differentially expresses activated cell-cycle genes.

The gene expression profiling results prompted studies to determine whether human MLLr LSCs are quiescent as suggested for most cancer stem cells or cycling, non-quiescent cells. Cell-cycle status was quantified by flow cytometry based on Hoechst and Pyronin Y staining in human MLLr AML cells comprising phenotypic subpopulations that differentially express CD34 and CD38. Consistent with previous studies, the CD34-CD38- subpopulation, which has been reported as LSC-enriched in AML, was the most quiescent of the phenotypic subpopulations (Figure 4A). However, further subdivision based on CD93 expression showed that despite the apparent quiescence of the overall CD34-CD38- fraction, cycling cells were highly enriched in the CD34-CD38- CD93+ subpopulation, consistent with the microarray results (Figures 4B and 4C). Prospective subfractionation of CD34+ CD38- CD93+ cells based on their cell-cycle status showed that G1 fraction (cycling) cells formed about 30-fold more colonies than G0 quiescent cells (Figure 4D). This contrasted with normal cord blood cells, which contained more CFCs in the G0 quiescent fraction compared to the G1 cycling fraction of Lin- quiescent cells (Figure 4F).

CD93 Is Essential for MLL Leukemogenesis

To assess whether CD93 may be more than a passive marker for cycling LSCs and thus required for maintenance of MLL leukemogenesis, its expression was knocked down in human MLLr leukemia cells using shRNA techniques. Efficient (>70%–80%) knockdown of CD93 expression dramatically reduced the clonogenic growth of human MLLr leukemia cells (Figures 5A and 5B) and promoted terminal leukemia cell differentiation compared with knockdown control (shLuc transduced) cells (Figure 5C).
or normal cord blood cells, which were unaffected (Figure 5B). Xenotransplantation experiments were performed to assess the potential in vivo dependence of primary human AML cells on CD93. Following lentiviral transduction of shRNAs and transplantation of unselected cells into NSG recipient mice, engraftment and disease progression were monitored by flow cytometry to quantify the relative proportions of human AML engraftment and disease progression were monitored by flow

**Figure 5. CD93 Is Required for MLL Leukemogenesis**

(A) Bar graph denotes transcript levels of CD93 as assayed by qRT-PCR of primary human MLLr leukemia cells transduced with lentiviral vectors encoding the indicated shRNAs.

(B) Bar graph shows the relative CFC activity of human MLLr AML cells and cord blood cells transduced with the indicated shRNAs. Error bars indicate SDs of five (human MLLr AML) and three (cord blood) independent experiments. *p < 0.0001.

(C) May-Grunwald-Giemsa-stained cytopsins show the morphologic features of human MLLr AML cells expressing the indicated shRNAs after methylcellulose culture (day 12).

(D) Graphs show the relative numbers of human leukemia cells transduced with the indicated shRNAs present in the BM of NSG recipient mice after seven (MLLr) or nine (non-MLLr) weeks post-transplantation, respectively. Data shown are representative of two cases each of MLLr and non-MLLr AMLs. Error bars indicate SDs of three independent mice.

**CD93 Is Required for Initiation and Establishment of Hox-Associated Leukemia**

The role of CD93 in MLL leukemogenesis was further investigated using a murine model that recapitulates the features of human MLLr leukemia. Similar to human MLLr AML, LSCs were enriched in the CD93+ subpopulation (Figure S6) and CD93 knockdown substantially decreased clonogenicity and growth of MLL leukemia cells in vitro (Figures 6A–6C). The requirement for CD93 appeared to be selective for myeloid cells transformed by oncogenes that dysregulate the Hox pathway including MLL and NUP98-HOXA9 (Figure 6D). CD93 knockdown colonies were smaller, more diffuse, and composed of morphologically more differentiated cells, supporting a role for CD93 in promoting maturation arrest and self-renewal (Figure 6E). CD93 knockdown AML cells were unable to induce leukemia in secondary transplant assays (Figure 6F) consistent with their more differentiated phenotype (lower c-Kit and higher Mac1 and Gr-1 expression) (Figure 6G). Together, these data demonstrate that CD93 is an essential regulator that selectively maintains MLL LSC oncogenic potential through enhanced self-renewal and maturation arrest.

**CD93 Regulates LSC Self-Renewal by Suppression of Cdkn2b in MLL Leukemia**

To assess the mechanistic role of CD93 in regulating LSC self-renewal, AML cells were analyzed by flow cytometry for cell-cycle status following CD93 knockdown. Both human and murine knockout AML cells were more quiescent (increased G0 cells) compared with control cells, suggesting that CD93 plays a crucial role in modulating cell cycle in MLL leukemia cells (Figures 7A–7C). Notably, qRT-PCR analysis of candidate cell-cycle regulators demonstrated substantial upregulation of the cell-cycle inhibitor Cdkn2b ($\alpha_{15\text{ (inlay)}}$) in CD93 knockdown versus control AML cells (Figures 7D and 7E). These results suggested a potential model whereby CD93 may functionally suppress Cdkn2b expression and prevent cell-cycle quiescence to promote LSC self-renewal. To test this model, we attempted to bypass the cell-cycle suppression caused by CD93 knockdown using cells from Cdkn2b$^{\text{−/−}}$ mice (Figure 7F). MLL-transformed cells deficient for Cdkn2b were significantly more resistant to the loss of clonogenicity induced by CD93 knockdown (Figure 7G) and were able to circumvent loss of maturation arrest as evidenced by colony morphology and cellular cytology (Figure 7H). Furthermore, Cdkn2b deficiency significantly attenuated the shift from G1 to G0 induced by CD93 knockdown (Figure 7I compared to Figure 7C) and rescued the leukemogenic potential of CD93 knockdown AML cells (Figure 7J compared to Figure 6F). Consistent with these results, overexpression of Cdkn2b in MLL leukemia cells induced morphologic differentiation and markedly suppressed oncogenic activity in colony-forming assays and transplanta
tion assays (Figure 7K and Figure S7). Thus, CD93 sustains LSC oncogenic potential of MLLr AML primarily through suppression of Cdkn2b.

**DISCUSSION**

We identified the cell-surface lectin CD93 as an LSC-specific surface molecule in MLL-rearranged AML. The various genetic
subtypes of AML display remarkable diversity in their developmental characteristics and pathological phenotypes, and a subtype-specific LSC marker has not been previously described. Furthermore, the LSCs marked by CD93 expression are predominantly quiescent and comprise a minor component of an otherwise actively cycling compartment of many AMLs, has not been shown to be essential or selectively enrich LSCs (van den Hurk et al., 2007). We hypothesize that when CD93 expression falls below a certain threshold CDKN2B is activated, cells exit the cycle, and they initiate differentiation out of the LSC compartment.

The association of CD93 with LSCs in MLLr AML, but not other genetic subtypes, likely reflects the fact that high CD93 expression correlates with elevated levels of MEIS1, an essential downstream effector in the transcriptional cascade subordinate to MLL oncogenes, although CD93 does not appear to be a direct target gene of MEIS1. Consistent with our results, CD93 was reported as one of 25 human candidate genes that discriminated LSCs from HSCs (Saito et al., 2010). Although early studies reported CD93 expression on normal HSCs (Danet et al., 2002), CD93-deficient mice are viable and display no major developmental abnormalities (Norsworthy et al., 2004), suggesting that CD93 is non-essential for HSC function, consistent with our observations that human cord blood stem/progenitor cells do not express and are not dependent on CD93, in contrast to MLLr LSCs.

Xenotransplantation assays showed that G1 phase CD34+CD38− “CD93−” cells retained strong LSC function in serial transplant assays, whereas G0 phase CD34+CD38− “CD93+” cells from MLLr AML inefficiently engrafted primary recipients. Since G0 reconstituted mice showed similar CD34/CD38 profiles and cell-cycle status to those of G1 reconstituted mice (Figure S5B), the cycling character of CD93+ LSCs was evident in their gene expression profiles and confirmed by xenotransplantation of FACS-sorted cycling versus quiescent cells from the same patient AML samples. The fact that LSCs in human MLLr AML are actively cycling contrasts with previous studies demonstrating that cancer stem cells are predominantly quiescent (Jordan et al., 2006) and our own analyses of non-MLLr AMLs (Table S5), but it is consistent with previous studies in a mouse model of MLL leukemia (Somervaille et al., 2009). Nevertheless, we cannot exclude the possibility of a rare quiescent LSC population in MLLr AML. Importantly, CD93 is more than a passive marker co-expressed on LSCs, but rather is required for LSC function, constituting an essential role for a lectin in LSC biology. By contrast, the C-type lectin-like molecule CLL-1, which is expressed on the whole blast compartment of many AMLs, has been shown to be essential or selectively enrich LSCs.
the G0 fraction was likely contaminated with G1 AML cells despite the extensive FACS procedure employed to prospectively isolate rare CD34/CD38/CD93 subpopulations based on their cell-cycle status. This is consistent with the long latency and reduced penetrance for AMLs induced by the enriched G0 subpopulation (Table S4).

CD93 is a C-type lectin transmembrane receptor that has been implicated in the modulation of phagocytosis, inflammation, and cell adhesion (Nepomuceno et al., 1997; Norsworthy et al., 2004; McGreal et al., 2002). It is expressed on cells involved in inflammation, including myeloid cells, endothelial cells, and platelets (Fonseca et al., 2001; Petrenko et al., 1999; Nepomuceno and Tenner, 1998). However, its molecular function has remained elusive. Although CD93 was originally identified as a receptor for complement molecule C1q (C1qR), subsequent studies reported that CD93 alone did not bind to C1q (Nepomuceno et al., 1997; McGreal et al., 2002). The CD93 requirement for AML cell maintenance in

Figure 7. CD93 Regulates the Cell Cycle through Suppression of Cdkn2b in MLL Leukemia

(A and B) Representative flow cytometry profiles are shown for Hoechst/Pyronin Y staining of murine MLL-AF9 AML (A) and human MLLr AML (B) cells expressing control or CD93 shRNAs 6 days following transduction.

(C) Bar graph shows cell-cycle stages of murine MLL-AF9 leukemia cells with (shCD93) or without (shLuc) CD93 knockdown after 6 days culture. Data are mean values from three independent experiments with statistically significant differences in G0 (p = 0.0231) and G1 (p = 0.0294) subpopulations.

(D) Bar graph shows relative levels of Cdkn2b transcript determined by qRT-PCR of murine MLL-AF9 leukemia cells expressing shLuc or shCD93. Results are the means of three leukemias and are expressed relative to shLuc control. *p = 0.0141.

(E) Bar graph shows relative levels of the indicated transcripts determined by qRT-PCR of murine MLL-AF9 leukemia cells expressing shLuc or shCD93. Results are the means of four leukemias in each category and are expressed relative to shLuc control. *p = 0.041.

(F) BM progenitor/stem cells harvested from wild-type or Cdkn2b−/− mice were transduced with the MLL-AF9 oncogene. Immortalized cells from the third to fifth round of replating were then transduced a second time with either shCD93 or shLuc-expressing lentivirus. FAC-sorted mCherry+/GFP+ cells were plated in methylcellulose for 5 days to determine CFC frequencies.

(G) Bar graph showing the relative CFC activity of murine MLL-AF9 immortalized cells derived from Cdkn2b−/− or Cdkn2b+/+ BM cells compared with (shCD93) or without (shLuc) CD93 knockdown. Error bars indicate SDs of five independent experiments. *p < 0.001.

(H) Representative images are shown for typical colonies and morphologies (May Grunwald Giemsa stain) of murine MLL-AF9 immortalized cells derived from Cdkn2b−/− or Cdkn2b+/+ BM cells with (shCD93) or without (shLuc) CD93 knockdown after methylcellulose culture (day 5).

(I) Bar graph shows cell-cycle stages of Cdkn2b−/− MLL-AF9-immortalized cells with (shCD93) or without (shLuc) CD93 knockdown after 6 days in culture. Data are representative of three independent experiments.

(J) Survival curves are shown for Cdkn2b−/− mice transplanted with murine MLL-AF9 AML cells (5,000 cells) transduced with shRNAs targeting CD93 or luciferase.

(K) Survival curves are shown for mice transplanted with murine MLL-AF9 AML cells transduced with Cdkn2b (n = 5) or control vector (n = 5).

See also Figure S7.
our culture assays suggests that its unknown ligand is present in vitro. One possibility is that AML cells may produce the ligand to sustain an autocrine loop, or alternatively CD93 may mediate essential homotypic cellular interactions to stimulate LSC self-renewal divisions. Future studies are warranted to address these possibilities.

Our studies link CD93 with cell-cycle regulation, and this occurs in part through suppression of the cell-cycle inhibitor p15ink4b. Loss of CDKN2B (p15ink4b) expression through mutation or epigenetic silencing plays a prominent role in leukemia pathogenesis. Hyper-methylation of the CDKN2B promoter occurs frequently in AML. (Drexler, 1998; Krug et al., 2002; Herman et al., 1996; Chim et al., 2001) and is correlated with poor prognosis (Wong et al., 2000; Shimamoto et al., 2005; Aggerholm et al., 2006; Teofili et al., 2003). Furthermore, Cdkn2b-deficient mice display increased susceptibility to retrovirus-induced AML and develop non-reactive monocytosis and hematologic features resembling chronic myelomonocytic leukemia (Wolff et al., 2003; Bies et al., 2010). Thus, CDKN2B is a tumor suppressor for myeloid leukemia; however, little is known about the signals driving its repression in leukemogenesis. Our studies indicate that lectin CD93, through currently undefined signaling pathways, provides a novel upstream mechanism to suppress CDKN2B expression and regulate LSC oncogenic potential in MLLr AML. CDKN2B is one of several downstream mediators of transforming growth factor β (TGF-β) signaling, which maintains a pool of quiescent HSCs (Oao et al., 2002), but the specific role of CDKN2B is not clear and MLLr LSCs are not malignant counterparts of HSCs, but rather, more downstream progenitors. Previous studies have shown that CDKN2B levels are undetectable in CD34+ cells and upregulated during normal and leukemic myeloid differentiation (Teofili et al., 2000). This suggests that suppression of LSC cell division in MLLr leukemia cells by cell-cycle inhibitors triggers the default outcome in the myeloid lineage, which is differentiation. This may account for why there appear to be few if any quiescent LSCs in MLLr leukemia. Recently it was reported that cell-cycle regulator CDK6, but not its functional homolog CDK4, is a crucial effector in MLLr AML (Placke et al., 2014). Since CDKN2B forms a complex with CDK6 to prevent its activation, our studies support a cell-cycle regulatory pathway in which CD93 suppresses CDKN2B, thereby freeing its target kinase CDK6 to drive cell-cycle progression and unrestricted LSC self-renewal.

In summary, we identified lectin CD93 as an LSC-specific cell-surface molecule in the genetic subtype of AML defined by MLL rearrangements. LSCs marked by CD93 are actively cycling in contrast to previous studies of quiescent cancer stem cells. LSCs functionally require CD93, which regulates their differentiation, self-renewal activity, and in vivo progression by modulating the cell cycle through CDKN2B. These results suggest that CD93 may be a useful candidate therapeutic target for anti-LSC therapy and prognostic marker for quantitation of minimal residual disease (MRD) for MLL-rearranged AML.

**EXPERIMENTAL PROCEDURES**

**Human Samples**

Human AML samples (Table S1) and cord blood cells were obtained from patients at the Stanford Medical Center with informed consent and institutional review board approval. Mononuclear cells were purified by Ficoll-density gradient centrifugation enrichment.

**Mice**

Inbred C57BL/6 mice were used for syngeneic transfection/transplantation experiments. NSG mice (NOD.Cg-PrkdcsndIl2rgtm1Wjl/SzJ) were obtained from the Jackson Laboratory and bred in a pathogen-free environment. Cdkn2b−/− mice were maintained on a C57BL/6 background. All experiments on mice in this study were performed with the approval of and in accordance with Stanford University’s Administrative Panel on Laboratory Animal Care.

**Cell Culture**

Immortalized mouse myeloid cells or leukemia cells were cultured in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS) and 20% WEHI conditioned medium or cytokines (10 ng/ml IL-6, 20 ng/ml SCF). 293T and Phoenix cells were cultured in DMEM supplemented with 10% FCS.

**In Vitro Colony Forming Assays**

Mouse cells were plated in methylcellulose medium (M3231, Stem Cell Technologies) with cytokines as described previously (Lavau et al., 1997) and counted after 5 days. Human cells were plated in H4535 (Stem Cell Technologies) and counted after 11–14 days of culture.

**Flow Cytometry**

Antibodies for flow cytometry were as follows. Anti-mouse antibodies used were CD93-PE-Cy7 or Biotin (AA4.1), Mac1/CD11b-PE or FITC (M1/70), Gr1-PE (RB6-8C5), B220-PE (RA3-6B2), F4/80-PE (BM8), NK1.1-PE (PK136), TER119-PE (TER-119), IL-7Rα-PE (SB14), CD34-PE (145-2C12), CD4-PE (GK1.5), CD8-PE (53-6.7), CD117-APC (2B8), Sca1-PE-Cy5 (D7), CD16/32-PE-Cy7 (2.4G3), and CD34-FITC (RAM34) (all from eBioscience). The lineage cocktail included the following mAbs: Mac1, Gr-1, B220, TER119, IL-7Rα, NK1.1, CD3, CD4, and CD8. Anti-human antibodies were CD34-APC (BG12) (BD Biosciences), CD38-PE-Cy7 (HT2) (eBioscience), CD93-PE or Biotin (R3) (eBioscience), CD45-APC or FITC (2D1 or H130) (eBioscience and BD Bioscience), CD3-PE-Cy5 (UCH1) (BD Biosciences) and CD64-PE (10.1) (BioLegend). Analyses and cell sorting experiments were performed using a FACS Vantage or FACS Aria II (BD Biosciences) equipped with Diva software (BD), and data were analyzed using FlowJo (Tree Star).

**DNA Constructs and Virus Production**

Retroviral constructs encoding HoxA9, Meis1, MLL-AF9, MLL-AF10, NUP98-HOXA9, and E2A-HLF were reported previously (Wong et al., 2007; Somervaille and Cleary, 2006; Iwasaki et al., 2005; Ayton and Cleary, 2003). The pSicoR lentiviral vector (Ventura et al., 2004) with either mCherry or GFP marker was used for knockdown studies. shRNAs were designed using pSicoOligomaker v1.5 (developed by A. Ventura, Jacks Laboratory) and sequences are listed in the Supplemental Experimental Procedures. Retrovirus and lentivirus production was performed as described previously (Wong et al., 2007; Somervaille et al., 2009).

**In Vivo Leukemogenesis Assays**

FACS-sorted mouse AML cells were transplanted intravenously into sublethally irradiated (4.5 Gy) C57BL/6 mice. Development of acute leukemia was confirmed by blood smear, peripheral blood leukocyte counts, FACS analysis, histology, or some combination thereof. For xenotransplant experiments, FACS-sorted human AML cells were transplanted into sublethally irradiated (2.5 Gy) NSG mice, which were monitored for development of AML. In some experiments, BM was analyzed for engraftment (hCD45+) at 2–4 months. See the Supplemental Experimental Procedures for more details of xenotransplant experiments.

**Microarray and Bioinformatics Analysis**

Total RNA was extracted from prospectively isolated cells using Trizol reagent (Invitrogen) followed by RNesasy MinElute Cleanup Kit (QIAGEN). Microarray experiments were performed in the Stanford PAN Facility with mouse Genome 430 2.0 arrays (Affymetrix) and human Gene 1.0 ST arrays (Affymetrix). Microarray data were normalized with Expression Console software (Affymetrix) using MA algorithm, then further normalized with dChip (Li and Wong, 2001).
GSEAs were performed using GSEA software (http://www.broad.mit.edu/gsea) with a t test metric for gene ranking and 1,000 data permutations. GO analysis was performed using MGI Gene Ontology Term Finder (http://informatics.jax.org/prototypes/GOTools/web-docs/MGI_Term_Finder.html).

**Cell-Cycle Analysis**

Cells were incubated with cell surface markers to enable our detection of phenotypic subpopulations and then incubated with Hoescht 33342 (Sigma-Aldrich) and Pyronin Y (Sigma-Aldrich) as previously described (Passegué et al., 2003).

**qRT-PCR**

RNA from sorted cell populations was purified using Trizol (Life Technologies). Real-time PCR was performed with TaqMan probes (Life Technologies) according to the manufacturer’s instructions. Real-time PCR was performed with TaqMan probes (Life Technologies) using β-Actin or β-ACTIN as internal control. TaqMan probes for the following mouse and human genes were purchased from Life Technologies: Cdkn1a (Mm00440239_g1), Meis1 (Mm00487664_m1), Cdkn1b (p21) (Mm01303209_m1), Cdkn1b (p27) (Mm00438167_g1), Cdkn2a (p19) (Mm01257348_m1), Cdkn2b (p15/16) (Mm00483241_m1), Cdkn2c (p18/19) (Mm00483243_m1), β-Actin (Mm00607939_s1); C193 (Hs00362607_m1), and β-Actin (HS01060665_g1). Primers for mouse p16ink4a were reported previously (Zhang et al., 2003) and purchased from Life Technologies.

**Statistical Analysis**

Statistics were calculated using Prism 6 software (GraphPad software, Inc). Data were presented as the mean ± SD. The significance of the differences between groups was determined via Student’s t test.

**ACCESSION NUMBERS**

The accession numbers for the expression data reported in this paper are GEO: GSE64773 and GEO: GSE64776.

**SUPPLEMENTAL INFORMATION**

Supplemental information for this article includes seven figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.08.008.

**AUTHOR CONTRIBUTIONS**

M.I. planned the study; conducted, performed, and interpreted experiments; and co-wrote the manuscript. M.L. provided patient AML samples. A.J.G. assisted with bioinformatics data interpretation. M.L.C. directed the studies and co-wrote the manuscript.

**ACKNOWLEDGMENTS**

We thank Cita Nicolas and Maria Ambus for technical support, Norm Cyr for graphical assistance, and Ravindra Majeti and Lawrence Okumoto for AML patient samples. We thank Nicholas Leeper and Juraj Bies for providing Cdkn2b knockout mice. We acknowledge support from the Children’s Health Initiative of the Packard Foundation, the California Institute for Regenerative Medicine, and Public Health Service (PHS) grant CA116606.

Received: January 15, 2015
Revised: May 28, 2015
Accepted: August 10, 2015
Published: September 17, 2015

**REFERENCES**


