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CD93 marks a non-quiescent human leukemia stem cell population and is required for development of MLL-rearranged acute myeloid leukemia

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SUMMARY

Leukemia stem cells (LSCs) are thought to share several properties with hematopoietic stem cells, including cell cycle quiescence and a capacity for self-renewal. These features are hypothesized to underlie leukemic initiation, progression, and relapse, and also complicate efforts to eradicate leukemia through therapeutic targeting of LSCs without adverse effects on HSCs. Here, we show that acute myeloid leukemias 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 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.

Keywords

CD93; leukemia stem cells; MLL; CDKN2B

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and six tables.

AUTHOR CONTRIBUTIONS

M.L. 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.

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 et al., 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., 2012), suggesting a potential function underlying 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., 2009; Jin et al., 2006; 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⁻ Sca1⁻ c-kit⁺ CD16/32⁺ CD34⁺) comparable to normal GMPs (Figure S1A, B), referred to as L-GMPs.

Gene expression profiling identified 2291 genes that were differentially expressed (at least 1.5 fold) in L-GMPs versus GMPs (1027 up-regulated versus 1264 down-regulated). Ten of the significantly up-regulated genes encoded candidate cell surface molecules (Figure S1C). These included CD93, a C-type lectin implicated in cellular adhesion, whose surface expression 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 Figure 1A-C). 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 comprised 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 nonclonogenic myeloid cells in the unfractionated 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 comprised 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 up-regulated versus 116 down-regulated). 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 (Figure 4B, C). 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–CD34+CD38– cells (Figure 4E). Xenotransplantation of prospectively isolated G0 versus G1 phase CD34+CD38–CD93+ cells from MLLr AML showed that G1 cells induced AML with higher incidence and shorter latency than G0 quiescent cells (Figure 4F and Table S4). In contrast, engrafting LSCs of non-MLLr AMLs were only present in the G0 subfraction of CD34+CD38– cells

(Table S5). Flow cytometry analysis of the xenograft AMLs demonstrated similar CD34/CD38 profiles and composition of cycling CD93+ G1 cells (Figure S5) as in primary patient AMLs (Figure 4A, B). The G1 phase CD34+CD38-CD93+ cells from xenograft MLLr AML serially engrafted secondary recipients indicating that the G1 cells have strong stem cell function and self-renew to produce themselves whereas G0 cells do not (Table S6). Taken together, these studies indicate that LSCs in human MLLr AML are highly enriched for actively cycling cells in contrast to the quiescence of non-MLLr LSCs and cancer stem cells in general.

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 (Figure 5A, B), 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 cells (hCD45+) that were knocked down for CD93 (mCherry+) in BM at 7-9 weeks post-transplant. CD93 knockdown markedly inhibited in vivo leukemia cell growth of MLLr AML with minimal effects on non-MLLr AML (Figure 5D). These results demonstrate that the cell surface lectin CD93, which marks cycling LSCs, contributes an essential function that sustains human MLL leukemogenesis.

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 (Figure 6A-C). The requirement for CD93 appeared to be selective for myeloid cells transformed by oncoproteins that dysregulate the Hox pathway including MLL and NUP98-HOXA9 (Figure 6D). CD93 knockdown colonies were smaller, more diffuse, and comprised 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 knockdown 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 (Figure 7A-C). Notably, quantitative PCR analysis of candidate cell cycle regulators demonstrated substantial upregulation of the cell cycle inhibitor *Cdkn2b* (*p15^{Ink4b}*) in CD93 knockdown versus control AML cells (Figure 7D, E). These results suggested a potential model wherein 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*^{-/-} 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 7C), and rescued the leukemogenic potential of CD93 knockdown AML cells (Figure 7J compared to Figure 6F). Consistent with these results, over-expression of *Cdkn2b* in MLL leukemia cells induced morphologic differentiation and markedly suppressed oncogenic activity in colony-forming assays and transplantation assays (Figure 7K and 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 cycling and comprise a minor component of an otherwise quiescent CD34+CD38- compartment of human AML. 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 is consistent with previous studies in a mouse model of MLL leukemia (Somerville 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 not been shown to be essential or selectively enrich LSCs (van Rhenen et al., 2007). We hypothesize that when CD93 expression falls below a certain threshold CDKN2B is activated, cells exit the cycle and initiate differentiation out of the LSC compartment.

The association of CD93 with LSCs in MLLr AML but not other genetic subtypes likely reflects that high CD93 expression correlates with elevated levels of MEIS1, an essential downstream effector in the transcriptional cascade subordinate to MLL oncoproteins, 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 as G1 reconstituted mice (Figure S5B), 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 et al., 1998). However, its molecular function has remained elusive. Although CD93 was originally identified as a receptor for complement molecule C1q (C1qRp), 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 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 p15^{Ink4b}. Loss of *CDKN2B* (p15^{Ink4b}) expression through mutation or epigenetic silencing plays a prominent role in leukemia pathogenesis. Hypermethylation 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 (Woff 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 (Dao 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 unrestrained LSC self-renewal.

In summary, we identified lectin CD93 as a 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 transduction/transplantation experiments. NSG mice (NOD.Cg-*Prkdc^{scid}IL2rg^{tm1Wjl}/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 Dulbecco's modified Eagle's medium (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 five 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-7R α -PE (SB14), CD3-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, Gr1, B220, TER119, IL-7R α , NK1.1, CD3, CD4, and CD8. Anti-human antibodies were: CD34-APC (8G12) (BD Biosciences), CD38-PE-Cy7 (HIT2) (eBioscience), CD93-PE or Biotin (R3) (eBioscience), CD45-APC or FITC (2D1 or H130) (eBioscience and BD Bioscience), CD3-PE-Cy5 (UCHT1) (BD Biosciences) and CD64-PE (10.1) (BioLegend). Analyses and cell sorting experiments were performed using a FACS Vantage or FACS Aria (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; Somerville et al., 2006; Iwasaki et al., 2005; Ayton et al., 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, Cambridge, MA) and sequences are listed in Supplemental Experimental Procedures. Retrovirus and lentivirus production was performed as described previously (Wong et al., 2007; Somerville et al., 2009).

In Vivo leukemogenesis assays

FACS-sorted mouse AML cells were transplanted intravenously into sublethally irradiated (4.5Gy) C57BL/6 mice. Development of acute leukemia was confirmed by blood smear, peripheral blood leukocyte counts, FACS analysis, and/or histology. For xenotransplant experiments, FACS-sorted human AML cells were transplanted into sublethally irradiated (2.5 Gy) NSG mice, which for monitored for development of AML. In some experiments, bone marrow was analyzed for engraftment (hCD45+) at 2-4 months. See 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 RNeasy 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 RMA algorithms, then further normalized with dChip (Li and Wong, 2001). Gene set enrichment analyses (GSEAs) were performed using GSEA software (<http://www.broad.mit.edu/gsea>) with a t-test metric for gene ranking and 1000 data permutations. Gene ontology analysis was performed using MGI Gene Ontology Term Finder (http://proto.informatics.jax.org/prototypes/GOTools/web-docs/MGI_Term_Finder.html).

Cell cycle analysis

Cells were incubated with cell surface markers to detect phenotypic subpopulations and then incubated with Hoechst 33342 (Sigma-Aldrich) and Pyronin Y (Sigma-Aldrich) as previously described (Passegue et al., 2005).

Quantitative RT-PCR

RNA from sorted cell populations was purified using Trizol (Life Technologies). cDNA was synthesized by SuperScript 1 First-Strand Synthesis System for RT-PCR (Life Technologies) according to manufacture'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: *Cd93* (Mm00440239_g1), *Meis1* (Mm00487664_m1), *Cdkn1a (p21)* (Mm01303209_m1), *Cdkn1b (p27)* (Mm00438167_g1), *Cdkn2a (p19^{Arf})* (Mm01257348_m1), *Cdkn2b (p15^{Ink4b})* (Mm00483241_m1), *Cdkn2c (p18^{Ink4c})* (Mm00483243_m1), β -Actin (Mm00607939_s1); *CD93* (Hs00362607_m1), β -ACTIN (HS01060665_g1). Primers for mouse *p16^{Ink4a}* 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 \pm standard deviation. The significance of the differences between groups was determined via Student's t test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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

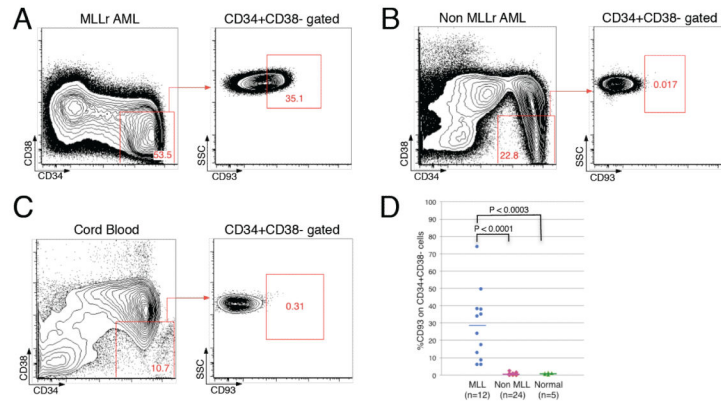


Figure 1. CD93 expression on human CD34+CD38- AML and cord blood cells

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

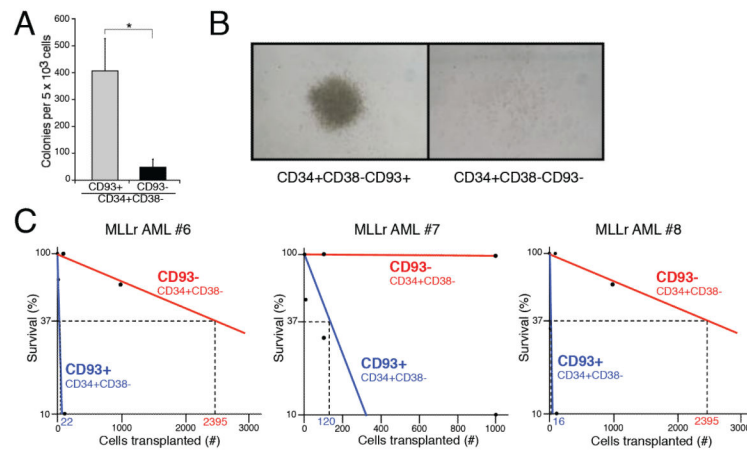


Figure 2. CD34+CD38-CD93+ human AML cells are highly enriched in LSC activity (A) Bar graph shows CFC frequencies for FACS sorted CD34+CD38-CD93+ or CD34+CD38-CD93- cells from primary MLLr AMLs (n = 5) plated in methylcellulose for 12 days. Data are the mean number of colonies (\pm SEM) per 5000 plated AML cells. *, p = 0.0006.

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

(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.

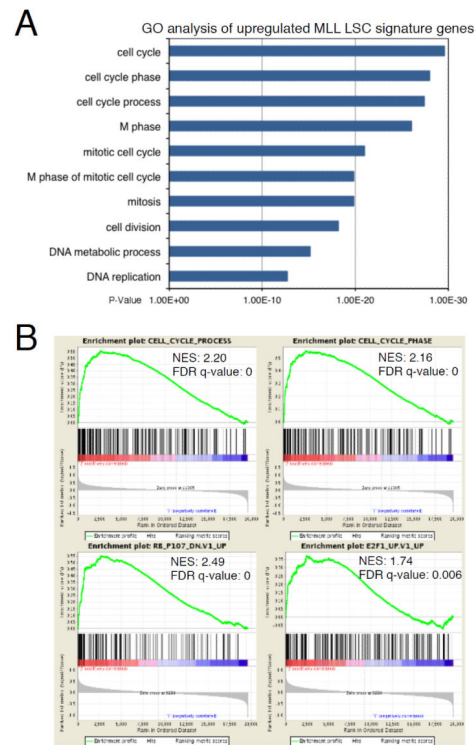


Figure 3. Gene expression profile of CD93+ LSC subpopulation strongly correlates with cell cycle related genes

(A) GO analysis of genes upregulated in the LSC-enriched CD34+CD38-CD93+ AML subpopulation based on their biological process annotations.

(B) GSEA plots show upregulation of cell cycle process-related genes in the LSC-enriched CD34+CD38-CD93+ AML subpopulation.

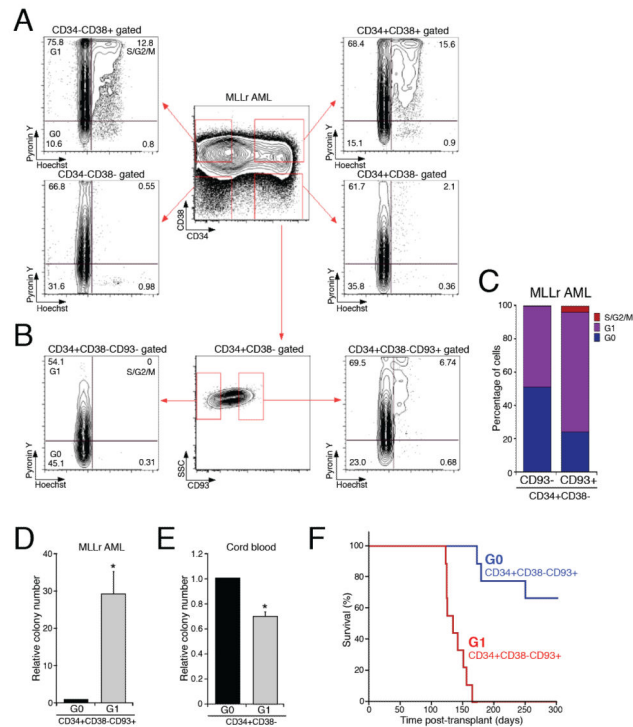


Figure 4. MLLr LSCs are enriched for G1 cycling cells, not G0 quiescent cells

(A, B) Representative flow cytometry plots show Hoechst/Pyronin Y staining profiles for the indicated AML cell subpopulations.

(C) Bar graph shows the cell cycle profiles for the indicated MLLr AML cell subpopulations as determined by flow cytometry. Data are mean values from three human MLLr AMLs with statistically significant differences in G0 ($p = 0.0133$) and G1 ($p = 0.0111$) subpopulations.

(D) Bar graph denotes the relative colony numbers generated by G0 versus G1 phase cells isolated by FACS from human MLLr CD34+CD38-CD93+ cells. Error bars indicate standard deviations of five independent experiments. *, $p = 0.0184$.

(E) Bar graph denotes the relative colony numbers generated by G0 versus G1 phase cells isolated by FACS from CD34+CD38- cord blood cells. Error bars indicate standard deviations of three independent experiments. *, $p = 0.0141$.

(F) Survival curves are shown for cohorts of mice transplanted with G0 or G1 phase cells (1000 cells) isolated from the CD34+CD38-CD93+ subpopulation of MLLr AML cells by FACS with Hoechst/Pyronin Y staining ($n = 9$ per cohort representing three human MLLr AML injected into three mice each). Acute leukemia was confirmed by flow cytometry (engraftment) and necropsy.

See also Figure S5 and Tables S4-S6.

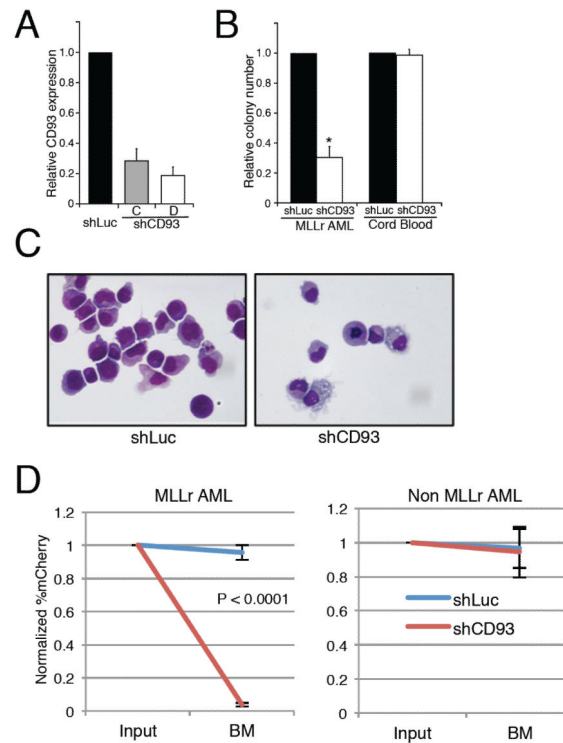


Figure 5. CD93 is required for MLL leukemogenesis

(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 standard deviations of five (human MLLr AML) and three (cord blood) independent experiments. *, $p < 0.0001$.

(C) May Grunwald Giemsa stained cytopreps 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 standard deviations of three independent mice.

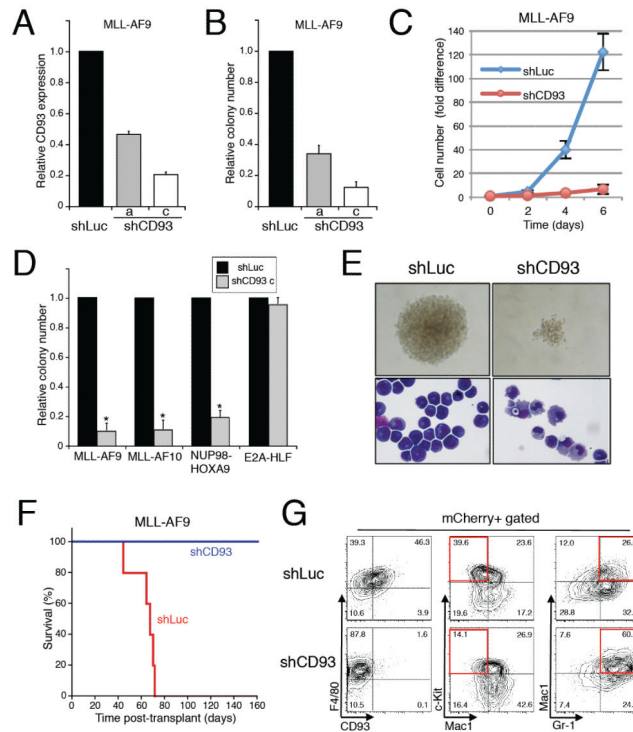


Figure 6. CD93 is required for murine Hox-associated leukemia development

(A) Bar graph shows relative CD93 transcript levels determined by qRT-PCR for primary murine MLL-AF9 leukemia cells transduced with the indicated shRNAs.

(B) Bar graph shows the relative CFC activity of murine MLL-AF9 AML cells transduced with the indicated shRNAs.

(C) Graph shows cell numbers (expressed as fold-change relative to day 0) for culture of murine MLL-AF9 leukemia cells expressing control or CD93 (construct c) shRNAs. Error bars indicate standard deviations of three independent experiments.

(D) Bar graph denotes the relative CFC activities of murine AML cells induced by the oncogenes listed below and expressing the indicated shRNAs. Error bars indicate standard deviations of at least three independent experiments. *, $p < 0.0001$.

(E) Representative images are shown for typical colonies and cellular morphology (May Grunwald Giemsa stain) for murine MLL-AF9 AML cells expressing CD93 or control shRNAs after methylcellulose culture (day 5).

(F) Survival curves are shown for mice transplanted with murine MLL-AF9 AML cells (5000 cells) transduced with shRNAs targeting CD93 ($n=5$) or luciferase ($n=5$).

(G) Flow cytometry profiles show representative expression levels for the indicated surface markers on murine MLL-AF9 AML cells expressing CD93 or control shRNAs after methylcellulose culture (day 5). Red boxes highlight significant changes in myeloid antigens.

See also Figure S6.

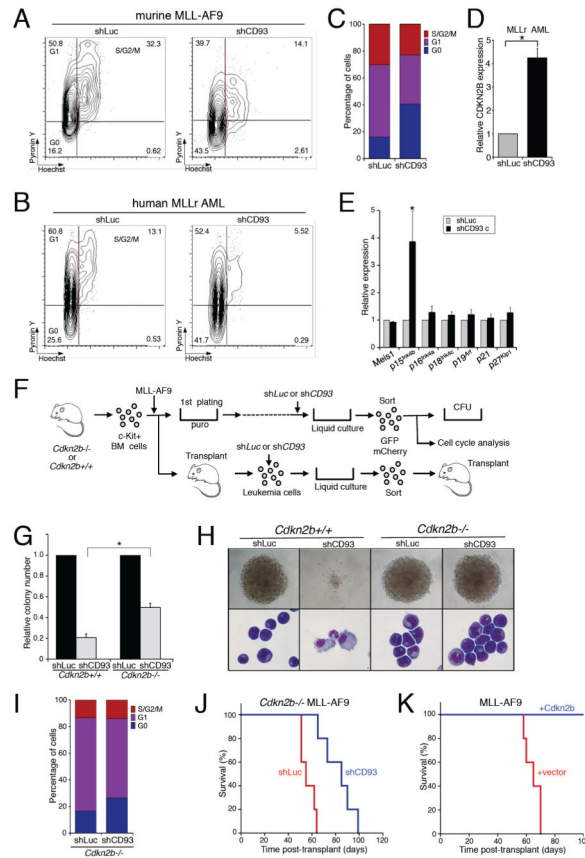


Figure 7. CD93 regulates the cell cycle through suppression of *Cdkn2b* in MLL leukemia

(A, 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 six 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 human MLLr AML 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. FACS-sorted mCherry⁺/GFP⁺ cells were plated in methylcellulose for five days to determine CFC frequencies.

(G) Bar graph shows 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 standard deviations 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 six days culture. Data are representative of three independent experiments.

(J) Survival curves are shown for *Cdkn2b*^{-/-} mice transplanted with murine MLL-AF9 AML cells (5000 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.