CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia

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Permanent cure of acute myeloid leukemia (AML) by chemotherapy alone remains elusive for most patients because of the inability to effectively eradicate leukemic stem cells (LSCs), the self-renewing component of the leukemia. To develop therapies that effectively target LSC, one potential strategy is to identify cell surface markers that can distinguish LSC from normal hematopoietic stem cells (HSCs). In this study, we employ a signal sequence trap strategy to isolate cell surface molecules expressed on human AML-LSC and find that CD96, which is a member of the Ig gene superfamily, is a promising candidate as an LSC-specific antigen. FACS analysis demonstrates that CD96 is expressed on the majority of $CD34^+CD38^-$ AML cells in many cases (74.0 ± 25.3% in 19 of 29 cases), whereas only a few (4.9 ± 1.6%) cells in the normal HSC-enriched population ($Lin^-CD34^+CD38^-CD90^+$) expressed CD96 weakly. To examine whether $CD96^+$ AML cells are enriched for LSC activity, we separated AML cells into $CD96^+$ and $CD96^-$ fractions and transplanted them into irradiated newborn $Rag2^{-/-}\gamma_c^{-/-}$ mice. In four of five samples, only $CD96^+$ cells showed significant levels of engraftment in bone marrow of the recipient mice. These results demonstrate that CD96 is a cell surface marker present on many AML-LSC and may serve as an LSC-specific therapeutic target.

hematopoietic stem cell

The cancer stem cell hypothesis holds that cancers are composed of a subset of cells that have the unique ability to transplant disease as well as self-renew (1–4). This concept may radically alter approaches to cancer therapies. We have proposed a multistep model of leukemogenesis in which protooncogenic events short of activating the self-renewal process occur in the self-renewing hematopoietic stem cell (HSC) population (5). Therefore, the clonal progression of preleukemias likely occurs in a succession of HSC subclones until augmented or poorly regulated self-renewal pathways are activated, leading to the emergence of final stage leukemic stem cells (LSCs) usually at the level of a downstream progenitor (5). We previously showed that in human acute myeloid leukemia (AML) characterized by *t*(8;21), the LSCs reside in the $CD34^+CD90^-CD38^{lo}Lin^-$ fraction of progenitors, whereas pre-LSC, detected during hematological remission, are present in the $CD34^+CD90^+CD38^{lo}Lin^-$ fraction (6), which is typically enriched for HSC (7). These observations suggest that cell surface antigens that are expressed on progenitor cells, but not on normal HSC, are possibly expressed on AML-LSC. Nevertheless, cell surface markers that can distinguish AML-LSC from normal HSC are still lacking, except for the IL-3Rα chain (CD123) (8).

One promising strategy for therapeutic targeting of LSC is immunotherapy with monoclonal antibodies (9). In fact, different antibodies or immunoconjugates, including CD20 (10), CD33 (11), and CD52 (12), have been successfully used for the treatment of hematologic malignancies. Previous studies have pointed to antibody-dependent cell-mediated cytotoxicity (ADCC) and FcR-mediated phagocytosis by the macrophage lineage as important mechanisms of activity for these antibody therapies (13). Therefore, when a cell surface antigen specific for LSC is identified, depleting antibodies against it can be potentially developed as therapeutics. In addition, cytotoxic substances such as chemotherapeutics, radioimmunoconjugates, or toxins can be coupled to enhance efficacy.

To identify cell surface markers selectively expressed on AML-LSC, we applied a signal sequence trap PCR method (14) to highly purified $CD34^+CD38^-$ AML cells, a fraction enriched in AML-LSC activity (15). We find that CD96 is selectively expressed in AML-LSC. CD96, which is also known as Tactile, is a member of the Ig gene superfamily and was first identified as a gene expressed in activated T cells (16). Furthermore, we demonstrate that $CD96^+$, but not $CD96^-$, AML cells can engraft immunodeficient mice (17). Taken together, CD96 is a marker for AML-LSC and therefore a potential target for LSC-specific therapy.

Results

Identification of CD96 as an AML-LSC-Specific Marker. To identify cell-surface molecules expressed on AML-LSC, we used the signal sequence trap PCR screening method, which allows for the detection of mRNAs that contain signal sequences by virtue of their ability to redirect a constitutively active mutant of c-mpl to the cell membrane, and thereby induce IL-3-independent growth of Ba/F3 cells (14) (Fig. 1*A*). We initially applied this method to amplified cDNA from 3.6×10^4 FACS-purified $CD34^+CD38^-$ AML cells (FAB classification: M2). Completion of the screen resulted in the identification of 33 independent genes [supporting information (SI) Table 2; 31 known, 2 unknown]. We then used an unamplified cDNA library prepared from 3×10^7 FACS-purified $CD34^+CD38^-$ cells from another AML (M2) sample and identified 41 genes (SI Table 2; 36 known, 5 unknown). Candidate AML-LSC marker genes were selected from these two data sets by excluding those genes that would not be expected to effectively discriminate AML-LSC from HSC, such as CD45. The expression levels of the

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Conflict of interest statement: I.L.W. was a member of the scientific advisory board of Amgen and owns significant Amgen stock. I.L.W. is also a cofounder and director of Stem Cells, Inc., and cofounded Cellerant, Inc. N.H. and I.L.W. have applied for a U.S. patent entitled "Identification of Cell Surface Marker for Acute Myeloid Leukemia Stem Cells" through the Stanford University Office of Technology and Licensing.

Abbreviations: AML, acute myeloid leukemia; HSC, hematopoietic stem cell; LSC, leukemic stem cell; NK, natural killer.

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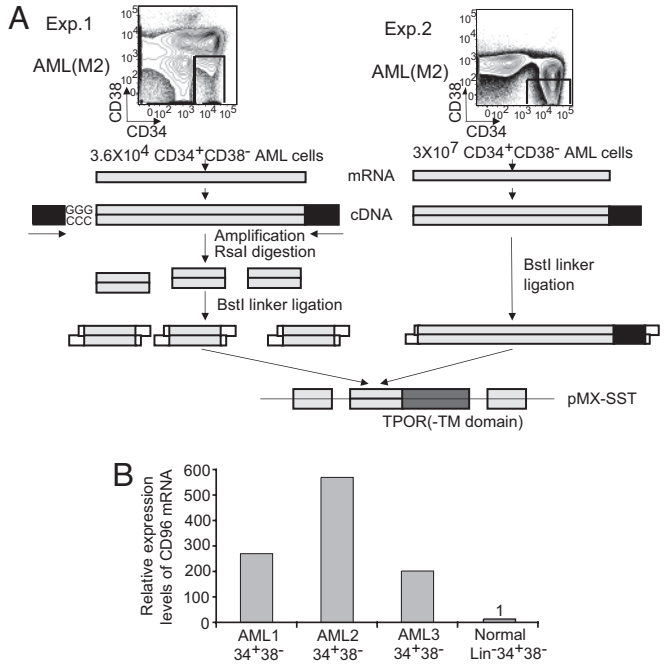

Fig. 1. SST screening of $CD34^+CD38^-$ AML-LSCs. (*A*) The scheme of the SST screening of $CD34^+CD38^-$ AML-LSCs. Full-length cDNA, either digested into pieces (experiment 1, *Left*) or undigested (experiment 2, *Right*), was ligated with a BstXI linker and subcloned into pMX-SST vector. TPOR, thrombopoietin receptor (a constitutively active mutant); TM, transmembrane. (*B*) CD96 mRNA expression level in $CD34^+CD38^-$ cells from three different AML (M2) samples and normal $CD34^+CD38^-Lin^-$ cells. The expression levels are shown relative to those in normal $CD34^+CD38^-Lin^-$ BM cells.

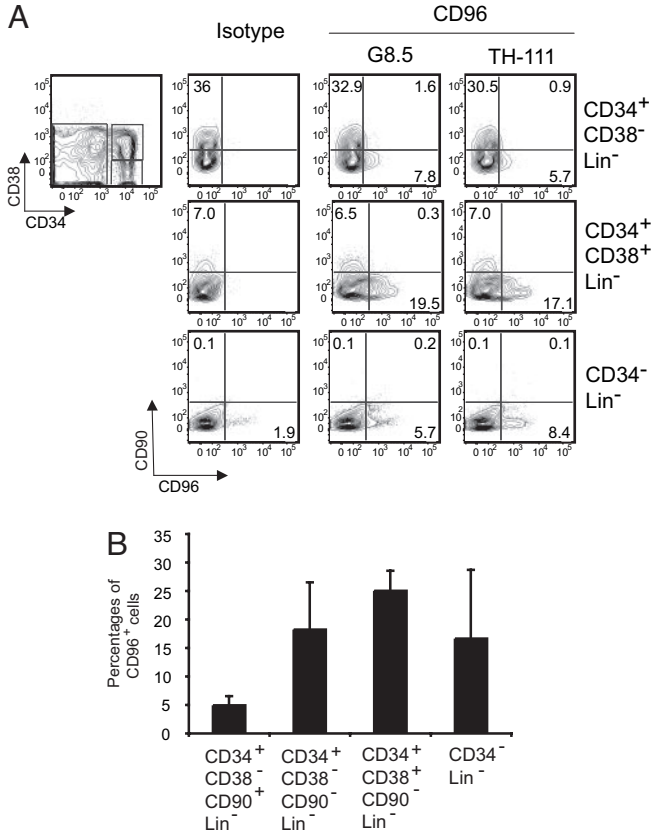

Fig. 2. CD96 expression in normal BM cells. (*A*) Lin^- BM cells were separated into subpopulations according to the expression of CD34 and CD38 and then analyzed for CD90 and CD96 expression, with the anti-CD96 clones, G8.5 or TH-111. Numbers represent the percentages of cells in the gated populations. Representative data from three different normal BM samples are shown. (*B*) Summary of the analysis of CD96 expression in normal Lin^- BM cells ($n = 3$). Error bars show the SD.

35 selected genes were measured by quantitative RT-PCR in $CD34^+CD38^-$ cells isolated from three different AML (M2) samples, as well as normal bone marrow (BM)-derived HSC/progenitors ($Lin^-CD34^+CD38^-$). Among the genes tested, only CD96 was found to be expressed at much higher levels in all three different $CD34^+CD38^-$ AML blasts compared to normal $Lin^-CD34^+CD38^-$ BM cells. CD96 mRNA expression levels in $CD34^+CD38^-$ blasts from three different AML samples were 270, 570, and 202 times higher than in normal $Lin^-CD34^+CD38^-$ BM cells (Fig. 1*B*).

CD96 Is Not Expressed by the Majority of Cells in the Normal HSC-Enriched Population. Two CD96 monoclonal antibodies, clone G8.5 (16) and clone TH-111 (18), were used to analyze CD96 expression in normal human adult HSCs and progenitor cells by flow cytometry. BM cells from three independent healthy adult donors were stained with CD96, CD34, CD38, CD90, and lineage markers and then analyzed by flow cytometry. Only $4.9 \pm 1.6\%$ of cells in the HSC-enriched population ($Lin^-CD34^+CD38^-CD90^+$) (7, 19) expressed CD96 weakly (Fig. 2 *A* and *B*). In other non-HSC populations, such as $Lin^-CD34^+CD38^-CD90^-$, $Lin^-CD34^+CD38^+$, and Lin^-CD34^-, CD96 was detected on $18.2 \pm 8.3\%$, $25.0 \pm 3.5\%$, and $16.6 \pm 12.1\%$ of the cells, respectively (Fig. 2 *A* and *B*). Colony-forming assays were performed by using FACS-purified $Lin^-CD34^+CD38^-CD90^-CD96^+$ as well as $Lin^-CD34^+CD38^-CD90^-CD96^-$ BM cells. Both fractions form GEMM, GM, and BFU-E colonies (SI Fig. 5), indicating that $Lin^-CD34^+CD38^-CD90^-CD96^+$ BM cells contain progenitor cells of myeloid/erythroid lineages.

CD96 Is Frequently Expressed in the AML-LSC Population. Next, we examined CD96 expression in 29 primary human AML samples. In 19 of 29 samples (65.5%), the percentage of CD96-positive cells in the $CD34^+CD38^-$ AML-LSC-enriched fraction (15) was significantly higher than in normal human BM $CD34^+CD38^-$ cells ($74.0 \pm 25.3\%$ vs. $12.2 \pm 2.7\%$) (Fig. 3 *A–C* and Table 1). CD96 is expressed almost exclusively in the $CD90^-$ subset (Fig. 3 *A* and *B* and SI Fig. 6). In the remaining 10 samples (34.5%), the frequency of CD96 expression in the $CD34^+CD38^-$ blasts was not increased compared to normal BM $CD34^+CD38^-$ cells (Table 1). To examine whether $CD34^+CD38^-CD96^+$ blasts express lineage markers, we analyzed three AML samples for the expression of CD96, in addition to numerous lineage markers (CD2, CD3, CD4, CD8, CD10, CD11b, CD14, CD19, CD20, CD56, and Glycophorin-A), as well as CD34, CD38, and CD90 (Fig. 3*D*). In all three samples, >85% of $CD34^+CD38^-CD96^+$ AML blasts did not express lineage markers.

Together these results indicate that CD96 is frequently expressed on $CD34^+CD38^-Lin^-CD90^-$ AML blasts, which are enriched for LSC activity and exclude HSC. When the AML samples were grouped according to their FAB category, CD96 expression in the AML-LSC population was more frequent in M2 compared to M0/M1 or M4/M5 samples (Fig. 3*C*).

CD96 Is Expressed on Functional AML-LSC. To determine whether CD96 is expressed on functional AML-LSC, we used FACS to fractionate primary human AML specimens into $CD96^+$ and $CD96^-$ populations, and we transplanted similar numbers of cells from each fraction into sublethally irradiated (100 cGy)

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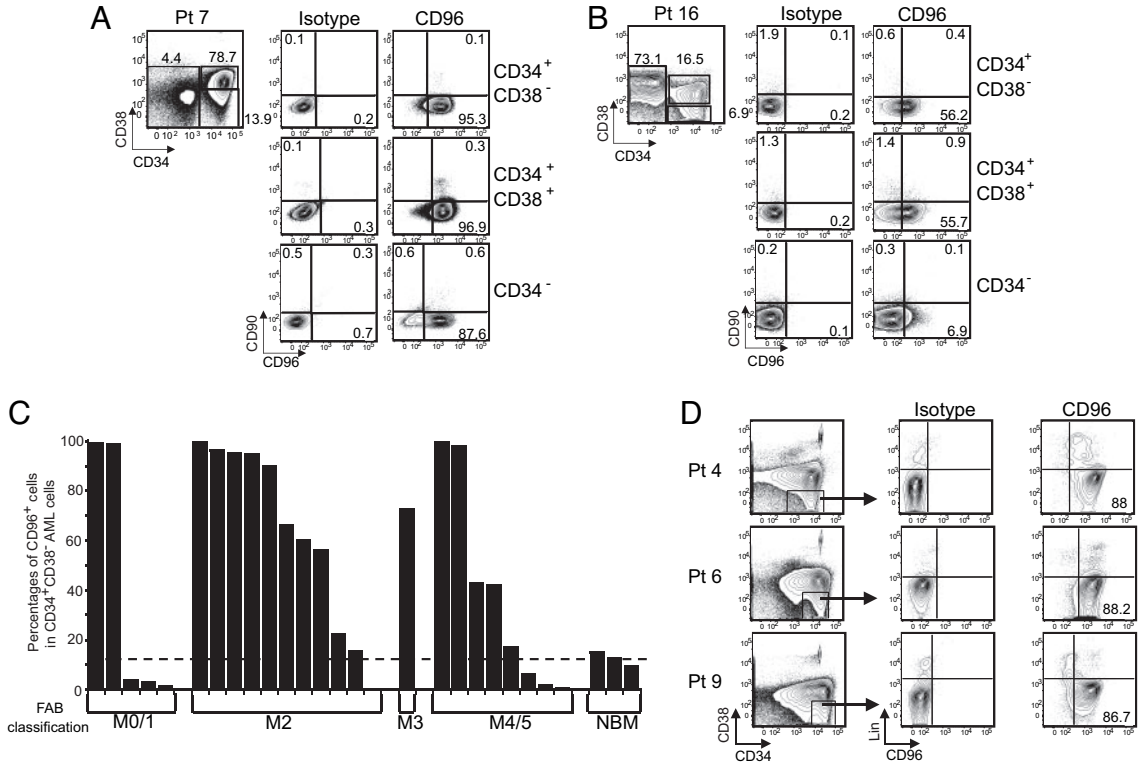

Fig. 3. CD96 expression in AML. (*A* and *B*) Expression profiles of CD34, CD38, CD90, and CD96 in AML samples. Cells were separated into subpopulations according to the expression of CD34 and CD38 and then analyzed for CD90 and CD96 expression. Representative data from AML samples that contain high (*A*) and intermediate (*B*) percentages of $CD96^+$ cells in the $CD34^+CD38^-$ population are shown. (*C*) Percentages of $CD96^+$ cells in the AML $CD34^+CD38^-$ population. Each bar represents a single AML sample. Pts 10, 15, 16, and 24 are not included in this graph because of the lack of information about FAB classification. The frequencies of $CD96^+$ cells in normal BM $CD34^+CD38^-$ cells are shown for comparison. The dotted line represents the average in normal BM $CD34^+CD38^-$ cells. (*D*) Expression of lineage markers and CD96 in $CD34^+CD38^-$ AML blasts. Numbers represent the percentage of $CD96^+Lin^-$ cells in $CD34^+CD38^-$ AML blasts.

newborn $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice via the facial vein (SI Table 3). This highly immunodeficient mouse strain lacks B, T, and natural killer (NK) cells (20), and newborn $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice support efficient engraftment of human AML (C.Y.P., R. Majeti, and I.L.W., manuscript in preparation). Transplanted mice were killed at 6 to 10 weeks after transplantation and analyzed for engraftment of human leukemia cells in BM. The results of a typical experiment are shown in Fig. 4*A*, with $CD96^+$ AML cells uniquely showing engraftment of human CD45 $(hCD45)^+$ cells. We confirmed that engrafted $hCD45^+$ cells were human myeloid leukemia blasts by measuring human CD13/CD14/CD33 expression (Fig. 4*A*) and/or evaluating Wright–Giemsa-stained cytospin preparations of the peripheral blood or BM cells (Fig. 4*B* and SI Fig. 7).

Transplantation experiments were performed by using five different AML samples (Fig. 4*D* and SI Table 3). For four of five cases, cells were separated into $CD96^+$ and $CD96^-$ populations regardless of the expression of CD34 or CD38 (Pts 5, 11, 14, 26). For one patient (Pt 3), cells were separated into $CD34^+CD38^-CD96^+$ and $CD34^+CD38^-CD96^-$ fractions. In four of five samples (Pts 3, 5, 11, 26), only $CD96^+$ AML cells showed significant levels of engraftment in the BM of recipient mice, whereas $CD96^-$ AML cells did not engraft. In the case of Pt 14, high levels of engraftment were observed with both $CD96^+$ and $CD96^-$ AML cells. It should be noted that for one specimen (Pt 26), the enrichment of LSC activity in the $CD96^+$ AML fraction was observed despite the low percentage of $CD34^+$ cells in this fraction. Finally, we analyzed CD96 expression on engrafted $hCD45^+$ cells from BM of mice transplanted with $CD96^+$ AML cells. As shown in Fig. 4*C*, transplantation of purified $CD96^+$ cells resulted in engraftment of both $CD96^+$ and $CD96^-$ cells, recapitulating the heterogeneity of CD96 expression in the primary AML specimen. Collectively, these results demonstrate that CD96 is expressed on functional LSC in human AML.

Discussion

CD96 has been reported to be expressed in T and NK cells, but not in B cells, granulocytes, monocytes, or RBCs (16, 18). In nonhematopoietic tissue, CD96 is expressed in the convoluted tubular epithelium of the kidney, the mucosal epithelium of the small and large intestines, and the vascular endothelium (18). It was also reported that CD96 is expressed in ≈30% of human AML samples, regardless of disease subtype, by examining whole BM cells from AML patients with immunohistochemical-staining (18); its expression was rare in the FAB-M5 subtype. In this study, we demonstrate that CD96 is frequently expressed in the $CD34^+CD38^-$ LSC population in >60% of the human primary AML samples examined. In some of the AML samples (e.g., Pt 16) (Fig. 3*B*), CD96 is highly expressed on the $CD34^+$ blasts, but at a lower frequency in $CD34^-$ blasts. Such samples may have been scored as negative for CD96 when unsorted leukemia cells were examined.

The xenotransplantation experiments reveal that CD96 is expressed on AML-LSC in all of the cases examined, and that in four of five cases, $CD96^+$AML cells are highly enriched for LSC activity compared to $CD96^-$ AML blasts. Furthermore, CD96 is not detectable on the majority of cells in the normal adult BM HSC-enriched population (7). Collectively, these results indicate that AML-LSC can be distinguished from normal HSC by the presence of CD96 expression. This finding suggests that CD96 may prove to be an excellent target for antibody therapy against LSC because hematopoietic progenitors are regenerated rapidly

Table 1. Patient characteristics

Patient no.	FAB classification	Cytogenetic abnormality	$CD96^+/CD34^+$ $CD38^-$, %	$CD96^+/CD34^+$ $CD38^+$, %	$CD96^+/CD34^-$, %	$CD96^+/Lin^-CD34^+CD38^-$, %
1	M5	FLT3 mutation	99.5	99.2	28.7	
2	M2	Inv(9)(p11q13)	99.2	95.1	51.8	
3	M1	Complex*	99.2	99.8	91.0	
4	M0/1	NA	97.8	99.8	32.9	99.6
5	M4Eo	Inv(16), FLT3D835	97.3	96.3	57.4	
6	M2	*t*(8;21), *t*(6;17)	97.4	98.6	69.9	94.9
7	M2	Normal karyotype	95.4	96.9	87.6	
8	M2	NA	93.4	98.7	33.6	
9	M2	NA	88.2	93.4	56.5	91.3
10	NA	FLT3ITD+	79.8	94.5	3.8	
11	M3	*t*(15;17) + complex†	72.7	91.6	88.4	
12	M2	NA	65.9	93.9	63.4	
13	M2	NA	59.9	51.6	59.8	
14	M2	*t*(8;21)	56.2	55.7	6.9	
15	NA	NA	51.1	37.8	63.2	
16	NA	6p+	43.3	59.5	17.0	
17	M5a	Normal karyotype	42.6	86.2	40.0	
18	M4Eo	del(16)(p13.1p13.3)	41.9	53.7	17.0	
19	M2	NA	22.1	21.9	5.8	20.7
20	M5	NA	16.8	26.2	3.1	0
21	M2	NA	15.1	14.2	6.8	18.3
22	M4/5a	13+	6.2	9.2	34.7	
23	M1	Normal karyotype	3.8	4.0	16.4	
24	NA	5q−	3.1	4.1	20.2	
25	M1	NA	2.8	1.1	11.2	
26	M4	NA	1.5	2.6	16.1	
27	M0/1	NA	1.1	3.1	66.0	
28	M5a	MLL translocation(FISH) 0.6 FLT3 mutation	1.2	3.4		
29	M2	NA	0	24.5	2.5	

NA, not available.

*46,XX,add(5)(q31),der(12)t(12;15)(q22~24;q11.2),der(12)add(12)(p13)?del(12)(q12), add(15)(q11.2)[cp3]/46,XX[cp17]

†46,XX, add(7)(q22), t(15,17)(q22:q12), add(x)(q22), add(3)(p11), add(4) (q21), der(9)add(9)(p13) del(9) (q?), *t*(11;20)(q13:q13)

from HSC. This therapy can be optimized by the development of CD96 antibodies that can induce cytotoxicity, such as ADCC, augmented macrophage phagocytosis, or complement-dependent cytotoxicity (21), although the expression of CD96 in T and NK cells, histiocytes, and nonhematopoietic cells has to be considered (18). In addition, *ex vivo* purging or FACS selection of stem cells with CD96 antibodies may be an avenue to pursue in autologous transplantation for AML patients.

An important observation made in our studies is that $CD96^+$ AML cells are enriched in LSC activity even in a sample that contained a low percentage of $CD96^+$ cells within the $CD34^+CD38^-$ population (Pt 26). These results indicate that the enrichment of LSC in $CD96^+$ AML is not simply a reflection of the enrichment of $CD34^+$ AML cells in the $CD96^+$ population. This finding suggests that CD96 may play a functional role in LSC biology. CD96 expressed on NK cells has been shown to bind to the polio virus receptor (CD155) and thereby mediate NK–target cell interactions such as those between NK and cancer cells (22). In addition, CD155 has been implicated as a human homologue of a protochordate histocompatibility gene (23). CD96 expressed on AML-LSC may also interact with its ligand on other cells in the BM, possibly niche cells for AML-LSC, and in these cells binding to the ligand cannot result in killer function, but may play a role in leukemia properties. More information may be obtained by examining the expression of CD155 in BM nonhematopoietic cells, such as osteoblasts or endothelial cells.

Although CD96 may play a functional role in AML-LSC biology, it may also have implications for the cell of origin for AML-LSC. One possibility is that AML-LSC may arise from the $Lin^-CD96^+CD34^+CD38^-$ $CD90^-$ population, ordinarily a non-self-renewing multipotent progenitor (R. Majeti, C.Y.P., and I.L.W., unpublished data); at a stage in leukemic progression in AML, this population may have gained the property of self-renewal. The lack of CD90 expression on most AML $CD34^+CD38^-$ cells (6, 24) suggests that the final leukemogenic event occurs in the $CD90^-$ population, which is downstream of the $CD90^+$ HSC population (7). In support of this hypothesis, we previously reported that AML1/ETO was expressed in $Lin^-CD34^+CD38^-CD90^+$ cells in remission, although CD90 expression was absent in the leukemia cells harboring the AML1/ETO translocation (6).

In summary, we provide evidence that CD96 is expressed on the majority of $Lin^-CD34^+CD38^-CD90^-$ AML blasts, the same population previously characterized as containing AML-LSC activity (6, 15, 25). Furthermore, we demonstrate that AML-LSC activity is highly enriched in the $CD96^+$ AML blast fraction. Because the majority of cells in the normal HSC population do not express CD96, these results suggest that CD96 is a marker of AML-LSC and a candidate therapeutic target.

Materials and Methods

Leukemia and Normal BM cells. Diagnostic AML BM cells were obtained after informed consent and with the approval of the research ethics committee at Stanford University or Osaka University. Normal BM cells from healthy volunteers were purchased from All Cells (Emeryville, CA).

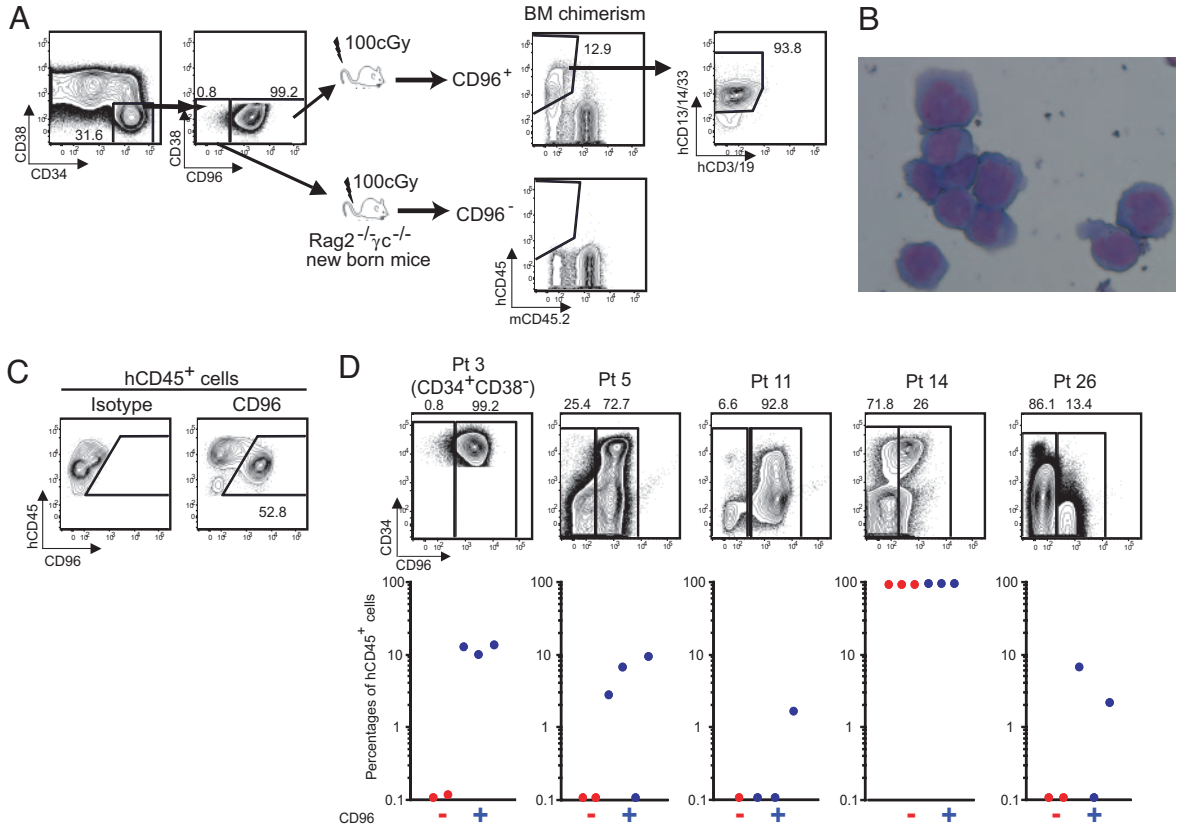

Fig. 4. CD96$^+$ AML cells are enriched in LSC activity. (*A*) Representative results of transplantation of CD34$^+$CD38$^-$CD96$^+$ or CD96$^-$ AML cells from Pt 3. (*Left*) Expression profiles of CD34, CD38, and CD96 and the sorting gates. CD96$^+$ or CD96$^-$ AML cells were transplanted into sublethally irradiated newborn Rag2$^{-/-}$ γ_c $^{-/-}$ mice. (*Right*) Analyses of human (hCD45$^+$) versus mouse (mouse CD45^{2+}) chimerism in the bone marrow (BM) at 6 weeks after transplant. (*B*) Wright–Giemsa stain of FACS-sorted hCD45$^+$ BM cells from an engrafted mouse transplanted with CD96$^+$ AML cells from Pt 3 (X630), showing that engrafted hCD45$^+$ cells have myeloblastic morphology. (*C*) CD96 expression on engrafted hCD45$^+$ cells in the BM of mice transplanted with CD96$^+$ AML cells from Pt 3. (*D*) (*Upper*) Expression profiles of CD34 and CD96 and the sorting gates for CD96$^+$ or CD96$^-$ populations for each AML sample. Note that the CD34$^+$CD38$^-$ population is pregated in the case of Pt 3. CD96$^+$ or CD96$^-$ AML cells were transplanted into irradiated newborn Rag2$^{-/-}$ γ_c $^{-/-}$ mice. Analyses of human engraftment are shown as the percentage of hCD45$^+$cells in the BM at 6 to 10 weeks after transplantation. Each dot corresponds to an individual mouse recipient.

Cell Separation, Immunophenotyping, and Sorting. BM mononuclear cells were isolated by Ficoll (GE Healthcare, Piscataway, NJ) gradient centrifugation and cryopreserved for later use. CD34-positive normal BM cells were enriched by using the MACS (magnetically activated cell sorting) CD34 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Single-cell suspensions were washed with PBS containing 2% FCS, incubated with 10% human AB serum for 20 min to prevent nonspecific antibody binding, and stained with either of two CD96 antibodies (G8.5; provided by A.M.K.) (16) or TH-111 (provided by M.G.) (18) or mouse IgG isotype control (eBioscience, San Diego, CA) for 30 min on ice. Cells were then washed and incubated with PE-conjugated anti-mouse IgG polyclonal antibody (eBioscience). Finally, cells were stained with Cy5-PE-conjugated lineage markers (CD2, CD3, CD4, CD8, CD10, CD11b, CD14, CD19, CD20, CD56, and Glycophorin-A) (Caltag, Carlsbad, CA), APC-conjugated CD34 (BD Bioscience, San Jose, CA), biotin-conjugated CD38 (Invitrogen, Carlsbad, CA), and FITC-conjugated CD90 (BD Bioscience) for 30 min on ice, followed by the incubation with streptoavidin-Cy7PE (eBioscience). Cells were resuspended in 1 μg/ml propidium iodide. Analysis and cell sorting were performed on a FACS Aria (Becton Dickinson, San Jose, CA). Data analysis was done with Flow Jo software (Tree Star, Ashland, OR).

Signal Sequence Trap Cloning. SST-REX cloning (14) was performed with slight modifications. CD34$^+$CD38$^-$ AML cells were FACS-purified, and total RNA was extracted by using the TRIzol reagent (Invitrogen). In the first experiment, cDNA was synthesized by using a PCR cDNA synthesis kit (SMART; Clontech, Palo Alto, CA) and oligo dT primer. cDNA was amplified by PCR and then digested with RsaI restriction enzyme and linked with a BstXI adaptor. cDNA fragments ranging from 0.5 to 2.0 kb were selected by electrophoresis on an agarose gel and subcloned into pMX-SST vector (a kind gift from Toshio Kitamura, Tokyo University, Tokyo, Japan). In the second experiment, full-length cDNA libraries were generated from FACS-purified CD34$^+$CD38$^-$ AML cells by using a cDNA library synthesis kit (Invitrogen) and random hexamers. cDNAs larger than 1 kb were selected by electrophoresis on an agarose gel and subcloned into pMX-SST vector. The SST-REX library was subjected to screening by transduction into BaF3 cells as previously described.

Quantitative RT-PCR. Quantitative RT-PCR analysis was performed by using SYBR Green on an ABI 7700 real-time PCR machine (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. The expression level of each gene was normalized to the β-actin expression level. PCR conditions and primer sequences are available on request.

Colony Assay. Methylcellulose culture assays were performed in Methocult H4334 (Stem Cell Technologies, Vancouver, BC,

Canada) according to the manufacturer's instructions. Colony numbers were enumerated and scored on day 14 of culture.

Transplantation into Newborn Rag2$^{-/-}$ $\gamma_c^{-/-}$ Mice. FACS-sorted AML cells were transplanted into newborn Rag2$^{-/-}$ γ_c $^{-/-}$ mice within 72 h after birth. Mice were irradiated with 100 cGy 4 to 24 h before transplantation and injected with sorted cells via the anterior facial vein. Mice were killed at 6 to 10 weeks of age to evaluate BM engraftment. Human chimerism was examined by staining with human CD45 antibody. Cells were stained with APC or Cy7PE-conjugated human CD45 (BD Bioscience), Alexa488-conjugated mouse CD45.2 (AL1–4A2), and Pacific blue or Cy5PE-conjugated anti-mouse Ter119 (eBioscience) and analyzed on a FACS Aria. In addition, PE-conjugated human CD13, CD14, and CD33 (BD Bioscience) and Cy5PE-conjugated human CD3 and CD19 (Caltag) were used for lineage analysis of engrafted human cells. When hCD45-positive cells were detected, their morphology was examined by Wright–Giemsa staining of a cytospin preparation.

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