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ARTICLE

CD97 is a critical regulator of acute myeloid leukemia stem cell function

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Despite significant efforts to improve therapies for acute myeloid leukemia (AML), clinical outcomes remain poor. Understanding the mechanisms that regulate the development and maintenance of leukemic stem cells (LSCs) is important to reveal new therapeutic opportunities. We have identified CD97, a member of the adhesion class of G protein–coupled receptors (GPCRs), as a frequently up-regulated antigen on AML blasts that is a critical regulator of blast function. High levels of CD97 correlate with poor prognosis, and silencing of CD97 reduces disease aggressiveness in vivo. These phenotypes are due to CD97's ability to promote proliferation, survival, and the maintenance of the undifferentiated state in leukemic blasts. Collectively, our data credential CD97 as a promising therapeutic target on LSCs in AML.

Introduction

Acute myeloid leukemia (AML) is initiated and maintained by leukemic stem cells (LSCs), which both self-renew and differentiate into non–self-renewing progeny that comprise the bulk of blasts (Bonnet and Dick, 1997). Despite recent advances in our understanding of the genetic origins of AML, clinical outcomes remain poor. While standard induction chemotherapy induces remission in most patients, the majority of patients eventually relapse and die from progressive disease (Lapidot et al., 1994; Ravandi and Estrov, 2006; Ishikawa et al., 2007). Although therapies targeting somatically acquired mutations and leukemogenic oncogenes are being pursued, these individual genetic lesions are present in only a subset of AML cases, and thus developing therapies with broader therapeutic potential is still an unrealized therapeutic goal (Rowe et al., 2005).

A number of cell surface proteins have been shown to be expressed at high levels on AML stem cells compared with normal hematopoietic stem cells (HSCs), including CD47 (Majeti et al., 2009), CD44 (Jin et al., 2006), CD96 (Hosen et al., 2007), TIM3 (Kikushige et al., 2010), CD123 (Jin et al., 2009), CD25 (Saito et al., 2010), and IL1RAP (Barreyro et al., 2012), and these antigens have become the focus of intense efforts to develop antibody-based or chimeric antigen receptor–T cell therapies (Majeti, 2011; Ågerstam et al., 2015; O'Hear et al., 2015). Despite the attention these antigens have received, data supporting their roles as cell-intrinsic regulators of LSCs are more limited, with IL1RAP supporting clonogenicity and increased cell death in AML cell lines (Barreyro et al., 2012) and TIM-3 supporting an autocrine stimulatory loop that regulates self-renewal of primary human LSCs (Kikushige et al., 2015). Thus, the efficacy of therapies targeting these antigens may be limited. Clinical trials of therapies targeting CD33 (Sekeres et al., 2013), CD123 (He et al., 2015a), and CD47 (https://clinicaltrials.gov/ct2/show/NCT02678338) are ongoing. While the outcomes of these studies are still pending, to date, therapies targeting LSC antigens have not yet been shown to significantly alter patient outcomes. Given that several of the targeted antigens in these trials are only expressed in a subset of primary AML (Jin et al., 2009; Barreyro et al., 2012), it is important to identify markers that are broadly and consistently expressed on LSCs to maximize the clinical impact of any single targeted therapy.

Previous transcriptomic studies have shown that mRNA or surface expression of the adhesion G protein–coupled receptor (GPCR) CD97 is increased in leukemic blasts, including immunophenotypically defined ($CD34^+$ or $CD34^+CD38^-$) LSC-enriched fractions (Saito et al., 2010; Bonardi et al., 2013; Mirkowska et al., 2013; Ho et al., 2016). CD97, encoded by the

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gene *ADGRE5*, is a founding member of the small EGF-TM7 subfamily in the GPCR adhesion class of cell surface proteins (Hamann et al., 2015). CD97 is expressed on multiple hematopoietic cell types including activated lymphocytes, granulocytes, monocytes, macrophages, and dendritic cells (Jaspars et al., 2001) and is also differentially expressed on murine hematopoietic stem/progenitor cells (HSPCs), with prior analysis showing that committed progenitor cells are largely $CD97^{-}$ while $CD97^{int}$ cells contain HSCs (van Pel et al., 2008). Studies have described a disease-modifying role for CD97 in several non-hematopoietic malignancies including breast (Park et al., 2013), thyroid (Ward et al., 2013), gastric (Liu et al., 2012), and prostate carcinoma (Ward et al., 2011), as well as glioblastoma (Safaee et al., 2013), by increasing the migration and invasive properties of cancer cells. A recent study also described a possible role of CD97 as a mediator of AML blast migration (Wobus et al., 2015); however, the functional role of CD97 in AML, especially in LSCs, has not been explored.

Here, we demonstrate that CD97 is frequently overexpressed in human AML and that it is a critical regulator of myeloid leukemogenesis in both mouse models of AML and human cell lines. CD97 promotes the proliferation and survival of leukemic cells, maintains their undifferentiated state, and is required for LSC maintenance. The mechanisms of CD97 action are at least partially mediated by activation of canonical GPCR pathways including the PI3K/Akt and MEK/ERK signaling pathways. Collectively, these data identify CD97 as a major regulator of LSC function in human AML and suggest it may be an important target for future therapeutic development.

Results

CD97 is frequently overexpressed in human AML

Identifying cell surface proteins expressed on AML blasts is of great interest, since such markers may be used in prognostic, diagnostic, and therapeutic settings (Majeti et al., 2009; Ågerstam et al., 2015; Kikushige et al., 2015). To determine whether CD97 may be expressed on LSCs, we assessed the expression of CD97 mRNA in human AML using data downloaded from Gene Expression Commons (https://gexc.riken.jp/models/7/genes/ADGRE5). CD97 mRNA was significantly overexpressed in LSC-enriched cells ($CD34^{+}CD38^{-}CD19^{-}CD3^{-}$) and leukemic progenitor cells ($CD34^{+}CD38^{+}CD19^{-}CD3^{-}$) from AML patient blasts compared with normal adult bone marrow (BM) HSCs and progenitor cells (Fig. 1 A). To confirm these findings, we evaluated CD97 cell surface expression in diagnostic AML patient blasts by flow cytometry. This study revealed that CD97 was increased on LSCs compared with cord blood (CB) HSCs ($Lin^{-}CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$) in all 30 patients tested. In contrast, the expression of other previously reported LSC markers was more variable and even decreased in some samples (Fig. 1 B); CD97 expression was similar between bulk and LSC-enriched ($CD34^{+}CD38^{-}$) blasts (Fig. S1 A). Consistent with our findings in AML patient blasts, CD97 was also expressed at significantly higher levels in all AML cell lines tested compared with CB as well as BM $CD34^{+}$ cell controls (Fig. S1 B). Comparison of CD97 surface expression by flow cytometry in paired diagnosis and relapse AML samples showed it was stably expressed (Fig. S1 C), suggesting it may be an excellent marker for minimal residual disease detection. To assess cell surface expression levels of CD97 on normal HSPCs, we evaluated both mouse and human cells. CD97 was not expressed at significant levels on HSCs over isotype controls but showed increasing expression in more differentiated progenitors as assessed by flow cytometry (Fig. S1, D and E). This pattern of expression was also confirmed at the mRNA expression level from publicly available information in https://gexc.riken.jp/models/3/genes/Adgre5.

Given the high frequency of CD97 expression on AML patient blasts, we assessed the correlation between CD97 expression and clinical outcomes. Evaluation of the Cancer Genome Atlas (TCGA) AML cohort (Ley et al., 2013) revealed that 75.5% of cases expressed higher normalized CD97 mRNA expression than normal $CD34^{+}$ CB cells, confirming frequent overexpression of CD97 transcripts in AML (unpublished data). Kaplan–Meier analysis showed that high CD97 mRNA expression is associated with inferior disease-free survival and overall survival (OS) when assessed based on median split (not depicted) as well as quartile split (Fig. 1 C). High CD97 expression also correlated with shorter OS in the intermediate cytogenetic risk group by Kaplan–Meier analysis (Fig. S1 F). Significant associations between high CD97 expression and somatic mutations were identified for fms-like tyrosine kinase 3–internal tandem duplication (FLT3-ITD), NPM1, and RUNX1 (Fig. S1 G, i). To determine if CD97 expression is independently prognostic, we performed univariate and multivariate analyses using Cox proportional hazards regression models for OS using the TCGA AML cohort including variables such as CD97 mRNA expression, age, sex, percentage of BM blasts, WBC count, cytogenetic risk group (favorable, intermediate, and poor), and specific somatic mutations. Compared with the lower quartile (the reference group), high levels of CD97 expression (median value >7,334) were significantly associated with inferior OS (univariate analysis: hazard ratio, 2.05; 95% confidence interval, 1.23–3.41; $P = 0.006$; multivariate analysis: hazard ratio, 3.04; 95% confidence interval, 1.74–5.34; $P < 0.001$; Fig. 1 D). Our evaluation of the TCGA data also confirmed previously reported correlations between TP53 mutations and inferior OS in our multivariate model (Fig. S1 G, ii). The prognostic value of CD97 expression as a single variable was confirmed by Kaplan–Meier analyses using additional AML transcriptome datasets (GSE10358, Tomasson et al., 2008; GSE37642, Fang et al., 2013; GSE12417, Metzeler et al., 2008; GSE6891, Verhaak et al., 2009; Fig. S1 H). Given the poorer outcomes of AML patients with $CD97^{high}$ expression, we sought to identify a gene signature associated with high CD97 expression and therefore selected samples with CD97 expression in the 90th percentile or higher ($CD97^{high}$) and those with CD97 expression in the 10th percentile or lower ($CD97^{low}$), using TCGA data. $CD97^{high}$ samples showed overexpression of genes enriched in functional pathways previously shown to be associated with either CD97 in nonleukemic contexts or AML pathogenesis, including inflammatory signaling, MAPK cascade activation, cytokine secretion, and chemotaxis (Jaspars et al., 2001; Lunghi et al., 2003; Ito et al., 2006; Milella et al., 2007;

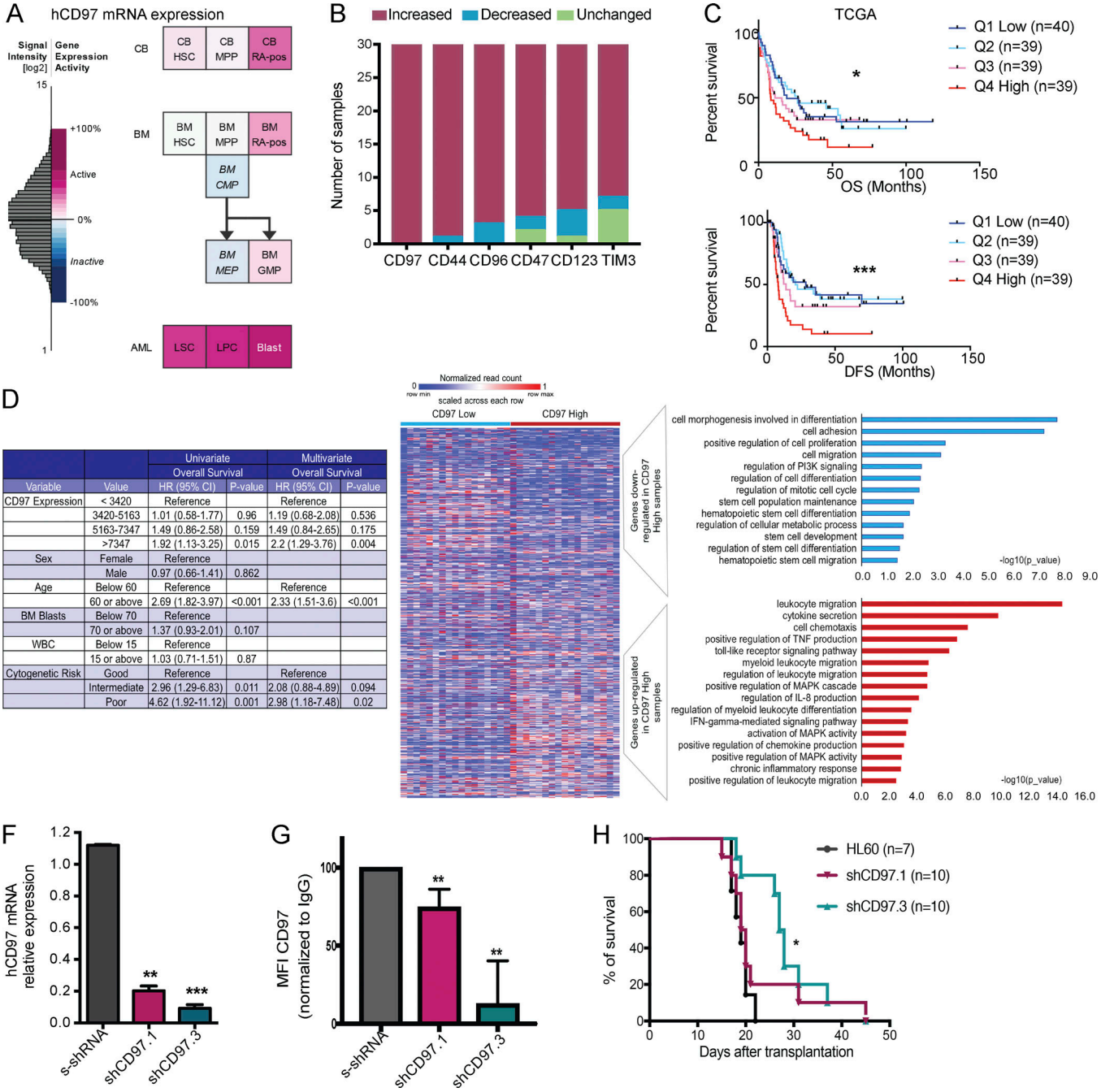


Figure 1. **CD97 is frequently overexpressed in AML. (A)** Human CD97 mRNA expression in CB, BM, or leukemic cells using data from Gene Expression Commons. LPC, leukemic progenitor cell. **(B)** CD97 and several other reported cell surface antigens reported to be present on LSCs were analyzed by flow cytometry in 30 diagnostic AML patient samples. Data shown represent cases that exhibited high expression in LSCs compared with CB HSCs (threshold was a ≥50% increase in mean fluorescence intensity [MFI] compared with CB). **(C)** Kaplan–Meier curves for disease-free survival (DFS) and OS based on analysis of the TCGA dataset for AML. **(D)** Univariate and multivariate Cox's proportional hazard analyses reveal that CD97 expression is associated with OS in the TCGA AML cohort. The multivariate model was additionally adjusted for DMNT3A, RUNX1, and TP53 (see Fig. S1 G, i). CI, confidence interval; HR, hazard ratio; WBC, white blood cell count. **(E)** Heatmap of mRNA expression, showing differentially expressed genes between TCGA CD97 mRNA high (top 10% of expressors) and CD97^{low} (bottom 10% of expressors) samples. Key GO biological processes associated with these genes are also shown. **(F)** Human CD97 mRNA quantification relative to GAPDH measured by RT-qPCR in HL60 cells infected with lentiviruses expressing two different shRNAs targeting CD97 (shCD97.1 and shCD97.3) or s-shRNA control. **(G)** CD97 cell surface protein expression (normalized to isotype control) was measured by flow cytometry in HL60 cells following shRNA-mediated KD of CD97. **(H)** Kaplan–Meier survival curve following transplantation of 8 × 10^6 CD97-KD (CD97.1 or CD97.3 shRNAs) or untransduced of s-shRNA control HL60 cells into NSG mice. Data shown as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figs. 1 E and S1 I; and Dataset 1). Prior reports have established the abundance of CD97 expression on activated lymphocytes, monocytes, macrophages, granulocytes, and various hematopoietic cell lines (Zhu et al., 2016). CD97 expression has also been reported to be associated with an inflammatory response (Yusuf and Scadden, 2009). Additionally, the functional significance of MAPK signaling in regulation of normal (Taniguchi Ishikawa et al., 2013) and malignant hematopoiesis (He et al., 2014) has been discussed at length in several previous reports, which collectively support a role for this signaling axis in the maintenance of HSC self-renewal capacity (Taniguchi Ishikawa et al., 2013) and regulation of cellular proliferation and apoptosis of AML blasts. All this evidence collectively asserts the relevance of association of these functional pathways with a molecular signature activated by overexpression of CD97. In contrast, genes overexpressed in CD97^{low} samples were associated with functions related to cellular differentiation, cell migration, and cell adhesion (Fig. 1 E and Dataset 1). These results were recapitulated when we analyzed gene sets in CD97^{high} samples segregated based on median value of CD97 expression (not depicted). Taken together, these studies demonstrate that CD97 is an independent prognostic marker of clinical outcomes in AML and suggest that it regulates multiple leukemic blast phenotypes.

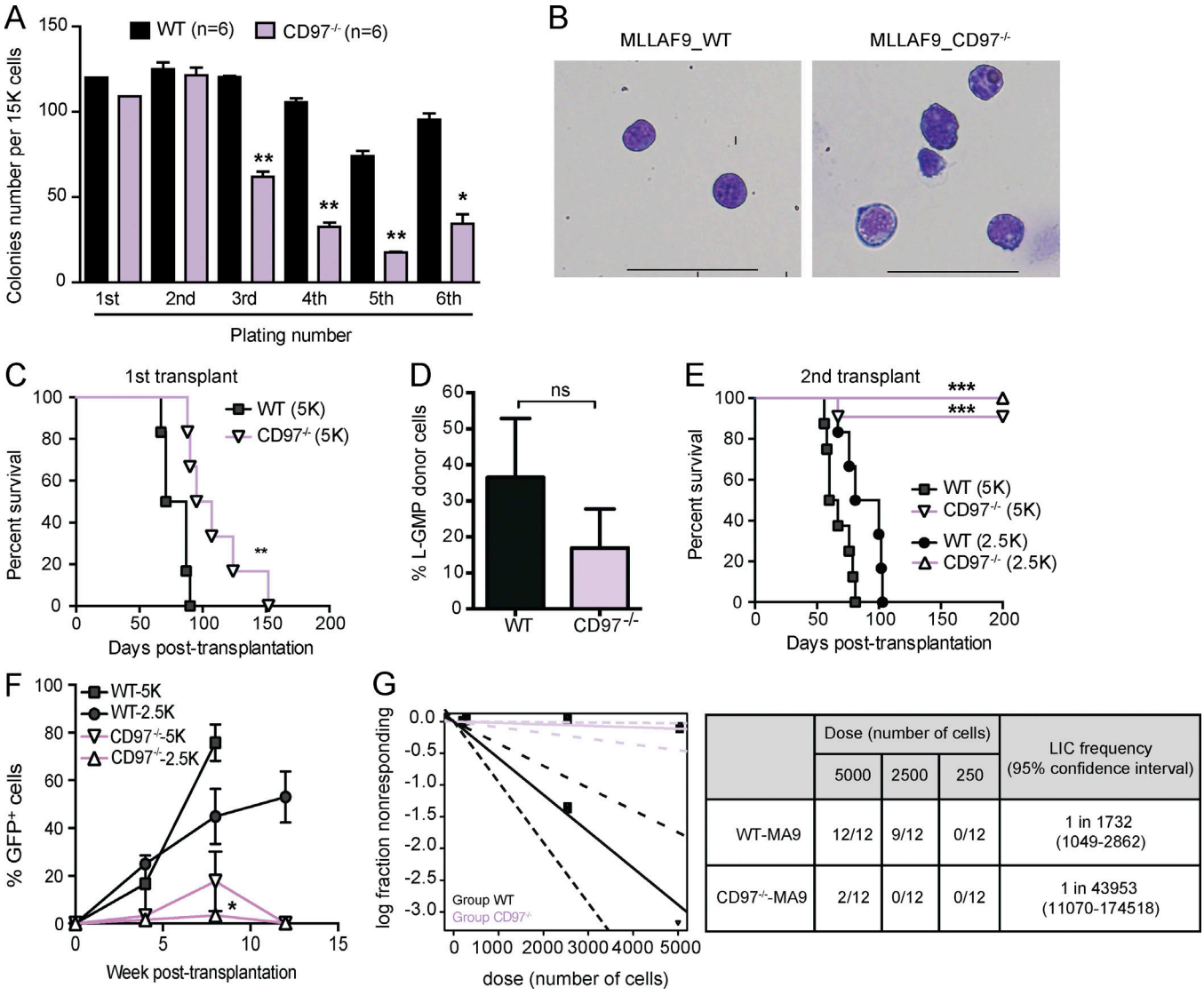
Since high CD97 expression is associated with poorer OS, we hypothesized that CD97 may regulate leukemic cell function and/or maintenance. To test this, we first knocked down CD97 (CD97-KD) in the human AML cell lines HL60 and MOLM-13 using lentiviral vectors expressing scramble control sequences (scrambled shRNA [s-shRNA]) or two shRNAs targeting CD97 (shCD97.1 and shCD97.3). Both shRNAs induced a significant decrease in CD97 mRNA expression (Fig. 1 F) as well as surface protein expression (Figs. 1 G and S1 J). To determine if CD97 regulates disease behavior in vivo, we transplanted adult immunodeficient NOD-scid-IL2Rγ common chain null (NSG) mice with control or CD97-KD cells. Mice transplanted with shCD97.3 KD HL60 cells survived significantly longer (28 d median survival) than mice transplanted with control cells (20 d median survival; P = 0.0012; Fig. 1 H). Similar findings were observed in xenografted mice transplanted with CD97-KD MOLM-13 cells (Fig. S1 K). Together, these data indicate that CD97 not only is a marker of AML cells, but also promotes disease aggressiveness.

CD97 is required for LSC function

To investigate whether CD97 plays a functional role in AML initiation, we purified HSPCs from WT and CD97-deficient (CD97$^{-/-}$) mice (Wang et al., 2007; Veninga et al., 2008) and transduced them with the leukemogenic oncogene, *MLL-AF9* (MA9; Krivtsov et al., 2006; Somervaille and Cleary, 2006). c-Kit$^+$ BM HSPCs from WT or CD97$^{-/-}$ mice were infected with a murine stem cell virus (MSCV)–driven retrovirus encoding MA9 and plated in methylcellulose. CD97$^{-/-}$-MA9 cells (GFP$^+$) showed a threefold reduction in serial replating capacity, consistent with a reduction in leukemic progenitor self-renewal (Fig. 2 A). CD97$^{-/-}$-MA9 transduced cells also exhibited cytological changes consistent with differentiation, including increased amounts of cytoplasm with vacuolization as well as nuclear folding and segmentation (Fig. 2 B). To confirm that CD97 is required for leukemic initiation in vivo, we transplanted MA9 transduced c-Kit$^+$ cells into sublethally irradiated congenic recipients and assessed leukemic engraftment. Mice transplanted with 1,000 WT-MA9 cells survived an average of 105 d, while CD97$^{-/-}$-MA9 cells failed to engraft (not depicted) or induce leukemia up to 200 d after transplant (Fig. S2 A). To determine whether these differences were due to a reduction in leukemia-initiating cell (LIC) frequency, we injected higher numbers of MA9 transduced cells. Mice transplanted with 5,000 WT-MA9 cells survived an average of 70 d, while CD97$^{-/-}$-MA9 cells survived 100 d (P = 0.0039; Fig. 2 C). Consistent with reduced levels of leukemic engraftment from CD97$^{-/-}$-MA9 transduced HSPCs, at the experimental endpoint, the mice injected with WT-MA9 cells showed a trend toward containing a higher percentage of total GFP$^+$ cells, leukemic granulocyte-monocyte progenitors (L-GMPs; Lin$^-$Sca-1$^+$c-Kit$^+$F$_c$γR$^+$CD34$^+$; Krivtsov et al., 2006; Fig. 2 D), and CD11b$^+$Gr1$^+$ cells in the BM (Fig. S2 B) than those receiving CD97$^{-/-}$-MA9 grafts.

To determine if CD97$^{-/-}$ blasts exhibit differences in LSC activity, we secondarily transplanted leukemic blasts at varying doses. WT-MA9 cells initiated secondary AML in a dose-dependent manner with transplants of 2,500 or 5,000 GFP$^+$ cells inducing death at 80 and 100 d, respectively (Fig. 2 E); similar dose-dependent differences in GFP$^+$ blast burden were also observed in the circulation (Fig. 2 F). In contrast, CD97$^{-/-}$-MA9 cells poorly engrafted secondary recipients with only 1 of 12 mice dead from leukemia at the 5,000 GFP$^+$ cell dose, verifying a critical role of CD97 in LSC self-renewal. Based on the secondary transplant experiments, the LIC frequency of CD97$^{-/-}$-MA9 blasts was calculated to be ∼25-fold lower than WT-MA9 blasts (Fig. 2 G).

As CD97 is highly expressed in the vast majority of human AML cases and its prognostic impact does not appear to be limited to specific genetic or cytogenetic subtypes of AML, we tested whether CD97 plays similar functional roles in other genetic subtypes of AML. Thus, we tested the leukemogenic potential of AML1-ETO9a (AE9a), a C-terminally truncated AML1-ETO protein of 575 amino acids considered to mimic human AML1-ETO (t(8;21)) AML when retrovirally expressed (Yan et al., 2006), in the CD97 deficient context. Transplantation of AE9a transduced WT c-Kit$^+$ cells (WT-AE9a) into lethally irradiated recipients resulted in leukemic death in 200 d, but mice injected with equal numbers of CD97$^{-/-}$ AE9a transduced cells (CD97$^{-/-}$-AE9a) survived up to 250 d without evidence of leukemia (P = 0.0013; Fig. S2 C). Similar to the MA9 model, absence of CD97 was associated with poor long-term engraftment, as WT-AE9a cells comprised 45% of total peripheral blood leukocytes, while CD97$^{-/-}$-AE9a cells were either absent or comprised <1% of leukocytes 16 wk after transplant (Fig. S2 D). Intriguingly, at early time points following transplant, there was no difference in engraftment levels observed between WT-AE9a and CD97$^{-/-}$-AE9a cells, suggesting CD97$^{-/-}$-AE9a LSC exhaustion at later time points. Consistent with this, at the time of death, 60% of WT-AE9a BM cells expressed c-Kit, while the rare residual CD97$^{-/-}$-AE9a cells (0.1% GFP$^+$ of total cells) were mostly myeloid CD11b$^+$ cells that were negative for c-Kit (not depicted). Morphological evaluation of BM cells confirmed



	Dose (number of cells)			LIC frequency (95% confidence interval)
	5000	2500	250	
WT-MA9	12/12	9/12	0/12	1 in 1732 (1049-2862)
CD97-/--MA9	2/12	0/12	0/12	1 in 43953 (11070-174518)

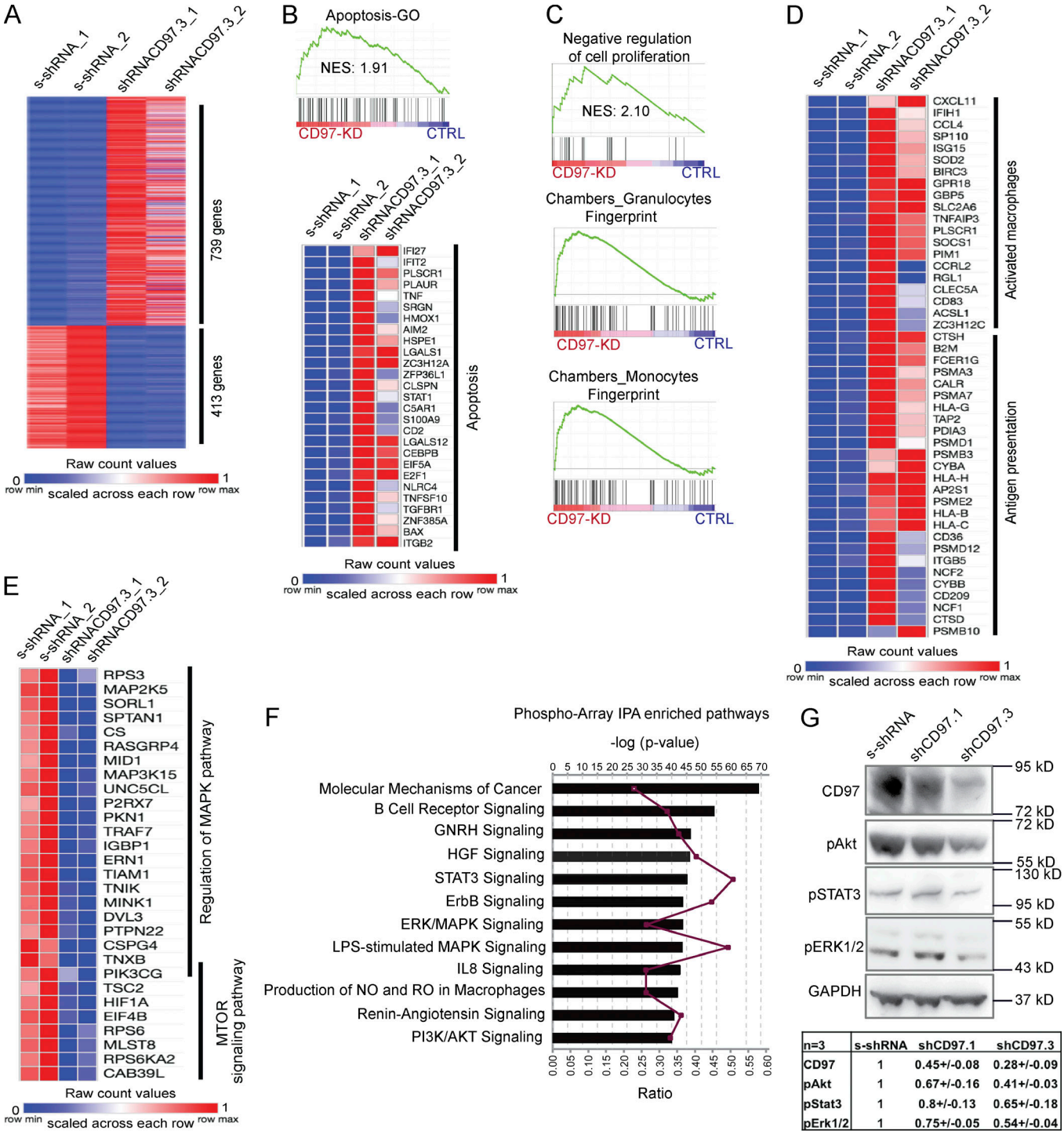
Figure 2. **CD97 is required to maintain LSC function in the MLL-AF9 murine model of AML.** c-Kit+ enriched BM cells from WT or CD97−/− mice were stably infected with a retrovirus encoding the MLL-AF9 oncogene coexpressing GFP. **(A)** Colony number generated from GFP+ cells serially plated in methylcellulose. **(B)** Wright-Giemsa stain of MLL-AF9 transduced WT and CD97−/− cells at the end of the sixth plating. Scale bar, 50 µm. **(C)** Congenic CD45.1+ mice were sublethally irradiated and transplanted with 5,000 GFP+ cells. Kaplan–Meier survival curves represented pooled results from two independent experiments (n = 6–8 in each group). **(D)** Flow cytometric evaluation of L-GMP (Lin−Sca-1+c-Kit+FcγR+CD34+) frequency among BM cells at the time of death. **(E)** Kaplan–Meier survival curves of mice secondarily transplanted with GFP+ cells from primary transplants. **(F)** Percentage of GFP+ cells in blood during the second transplantation. 2.5K WT versus CD97−/− leukemic grafts were statistically different. **(G)** Calculation of LIC frequency based on secondary transplantation of GFP+ leukemic cells. Data shown as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. ns, not significant between compared groups.

the immature nature of WT-AE9a engrafted cells, while CD97−/−-AE9a cells exhibited cytological features consistent with maturing granulocytes (Fig. S2 E). Taken together, these data demonstrate a critical role for CD97 in leukemic initiation and maintenance, the two key features that define LSCs.

To confirm that differences in homing potential do not contribute to the differences in leukemic engraftment, we evaluated engraftment immediately following transplantation with WT-MA9 and CD97−/−-MA9 cells. 4 d after retroviral transduction of Lin−c-Kit+Sca-1+ cells with MA9 (GFP+), GFP+c-Kit+Lin− cells were sorted, and equal numbers of cells (1,500) were transplanted into lethally irradiated recipient mice. 18 h later, the recipient mice were sacrificed, and the BM cells were isolated and examined by flow cytometry. No significant differences were found in the absolute number (Fig. S2 F) or frequency (Fig. S2 G) of WT-MA9 and CD97−/−-MA9 cells, indicating that CD97−/−-MA9 cells do not exhibit defects in BM homing activity.

Transcriptome analysis identifies functional pathways associated with CD97 expression

To understand the molecular mechanisms by which CD97 regulates AML blast function, we evaluated the cellular functions of the 1,121 differentially expressed genes (fold change ≥2, adjusted P value <0.01) identified in RNA sequencing (RNA-seq) data generated from WT and CD97-KD HL60 cells (Fig. 3 A and Dataset 1). Compared with control s-shRNA HL60 cells, CD97-KD



n=3	s-shRNA	shCD97.1	shCD97.3
CD97	1	0.45+/-0.08	0.28+/-0.09
pAkt	1	0.67+/-0.16	0.41+/-0.03
pStat3	1	0.8+/-0.13	0.65+/-0.18
pErk1/2	1	0.75+/-0.05	0.54+/-0.04

Figure 3. **CD97 activates multiple signal transduction pathways. (A)** Heatmap representing 1,152 genes differentially expressed by HL60 cells following infection by s-shRNA or shCD97.3 as determined by RNA-seq (cutoff log fold change < or >1 and P < 0.05). **(B)** Representative heatmap shows genes enriched in apoptosis biological processes in CD97-KD cells compared with s-shRNA, assessed by Gene Ontology Panther software (GO). **(C)** GSEA gene set for proliferation and granulocytes and monocytes fingerprint. CTRL, control. **(D)** Representative heatmap showing genes enriched in activated macrophages and antigen presentation, as assessed by GO. **(E)** Representative heatmap showing genes enriched in regulation of MAPK cascade and MTOR signaling pathway biological processes, as assessed by GO. See also Fig. S3. **(F)** Top pathways enriched among differentially phosphorylated and expressed proteins in s-shRNA and CD97-KD HL60 cells, as assessed by a phosphoprotein antibody array (Kinexus) using Ingenuity Pathways Analysis (IPA) software (see Table S1). GNRH, gonadotropin-releasing hormone; HGF, hepatocyte growth factor; RO, reactive oxygen. **(G)** Representative Western blot analysis performed on whole-cell lysates from HL60 s-shRNA or CD97-KD cells to assess the expression of ERK1/2 (Thr202/Tyr204), STAT3 (Tyr705), AKT (Ser473), and GAPDH (top). Relative mean values of quantitated bands from three independent experiments are shown ± SEM (bottom). NES, normalized enrichment score.

HL60 cells were significantly enriched in genes involved in apoptosis, including known apoptosis regulators such as Bcl-2–associated X protein, TNF family members, and TNF-related apoptosis-inducing ligand, as well as cell proliferation (Fig. 3 B), supporting a role of CD97 in promoting blast survival. CD97 silencing also induced differentiation, as genes associated with myeloid differentiation such as *CEBPB* and *S100A9*, as well as genes associated with granulocyte and monocyte differentiation fingerprint signatures (Harris et al., 2012), were induced in CD97-KD cells (Fig. 3 C). Multiple cytokines, cytokine receptors, chemokines, and genes involved in antigen presentation, including *TAP*, *B2M*, and several HLA genes, were up-regulated in CD97-KD cells, consistent with innate immune cell activation (Figs. 3 D and S3, A and B). Collectively, these data confirm that CD97 maintains the immature state of leukemic blasts and supports their survival.

To evaluate the consequences of CD97 loss on LSC gene expression, we analyzed the transcriptomes of WT-MA9 and CD97$^{-/-}$-MA9 mouse LSCs (L-GMPs). 264 genes were differentially expressed (fold change ≥ 1.5, adjusted P value <0.01), in CD97$^{-/-}$ versus WT L-GMPs including multiple known MA9 target genes including *HOXA9* and *MEIS1* (Fig. S3 C). Similar to CD97-KD HL-60 cells, CD97$^{-/-}$ L-GMPs exhibited up-regulation of genes that promote cell death, proliferation, and differentiation (Fig. S3 D), and down-regulation of genes strongly associated with the regulation of cell migration, cell cycle, and the MAPK signaling (Fig. S3 E). Together, these results confirm that CD97 is associated with cell survival, apoptosis, and maintenance of an undifferentiated phenotype, all required for LSC function.

CD97 activates multiple signaling pathways

GPCRs couple to one or more heterotrimeric G proteins to activate multiple cell signaling pathways including PKA- and ERK-dependent signaling (Gs subtype); SRC, PI3K, RAC–MEK–ERK, CDC42–PAK, and Rho-mediated signals (Gαi); and phospholipase C (Lappano and Maggiolini, 2011). Thus, CD97 is expected to mediate its function through multiple signal transduction pathways, although CD97-dependent downstream signaling pathways are poorly understood and have not been investigated in AML. To identify CD97-dependent pathways in AML, we analyzed the enrichment of genes down-regulated in CD97-KD cells in different gene set enrichment analysis (GSEA) functional signatures and Gene Ontology (GO) biological processes. Several genes encoding mediators of MAPK cascade were down-regulated in CD97-KD cells compared with s-shRNA infected cells, with decreased expression of some of the direct mediators of the cascade such as MAP2K5, MAP3K15, and the oncogene RAS, as well as downstream targets such as TIAM, SPTAN1, or MINK1 (Fig. 3 E). Moreover, we observed decreased expression of genes that encode components of the mammalian target of rapamycin (MTOR) signaling pathway such as TSC2, EIF4B, and RPS6. These analyses suggest that alterations in cell growth, survival, and differentiation in CD97-KD AML cells may be modulated by specific signaling cascades, such as MAPK and MTOR.

To directly examine the signaling pathways activated by CD97, we performed a phosphorylation-antibody array screen. Ingenuity Pathway Analysis revealed that CD97-KD decreased phosphorylation of members of the MAPK and PI3K signaling cascades (Fig. 3 F). Indeed, we observed a strong reduction of activating phosphorylation events on a significant number of signaling proteins, including members of the MAPK/ERK pathway (e.g., PKC isozymes, ERK1, and MAPKAPK2), Akt1 (S729), IGF1R, α-catenin, and heat shock proteins (e.g., HSP27 and HSP90b), among others (Table S1). In addition, several "activating" phosphorylation events were detected, most notably phosphorylation of STAT1 (Y701) and NF-κB p65 (S536), two signaling pathways strongly associated with differentiation (Bottero et al., 2006; Fang et al., 2013). We confirmed some of these signaling changes by Western blot analysis, showing that activating phosphorylation events on ERK 1/2 (Thr202/Tyr204), AKT (Ser473), and STAT3 (Tyr705) were decreased following CD97-KD (Fig. 3 G).

CD97 promotes AML cell proliferation and survival

To assess the relevance of our findings to AML cell function, we first determined whether CD97-KD affected AML growth and survival in vitro. Strikingly, CD97-KD HL60 cells grew approximately four times slower than the control cells transfected with s-shRNAs (Fig. 4 A); a similar trend was observed for CD97-KD MOLM13 cells (Fig. S4 A). This reduction in CD97-KD cell growth was associated with increased apoptosis, as demonstrated by increased expression of cleaved caspase-3 (Figs. 4 B and S4 B). CD97-KD cells did not exhibit differences in cell cycle status compared with control cells (Fig. 4 C), suggesting that CD97 affects survival rather than proliferation. Moreover, CD97$^{-/-}$-MA9 blasts exhibited significantly reduced growth ex vivo (Fig. 4 D) and displayed increased apoptosis compared with WT-MA9 cells, as demonstrated by a higher percentage of activated caspase-3–positive cells (Fig. 4 E).

CD97 inhibits AML blast differentiation

As CD97 expression is induced during normal myelomonocytic differentiation (McKnight and Gordon, 1998), we tested whether CD97 plays a similar role in AML blast differentiation. Using the human AML cell lines HL60 and U937, which can be induced to differentiate along the myelomonocytic lineage following all-trans retinoic acid (ATRA) treatment (Breitman et al., 1980), we cultured WT and CD97-KD HL60 cells for 4 d in the presence of 10 nM ATRA or 0.001% ethanol vehicle control (untreated). Flow cytometric analysis revealed that CD97-KD HL60 cells expressed higher levels of the myeloid differentiation antigens CD11b and CD15 both before and after ATRA treatment (Fig. 5 A). Similar results were obtained using U937 cells (Fig. S4 C). Cytological evaluation of Wright-Giemsa stains revealed decreased nuclear:cytoplasmic ratios, increased cell size, and cytoplasmic vacuolization with occasional cytoplasmic granules, confirming the induction of differentiation upon CD97-KD, both before and after treatment with ATRA (Fig. 5 B). In addition, mRNA expression of Itgam (CD11b) and Spi1 (PU.1), key factors associated with myeloid differentiation, were increased in CD97-KD cells compared with controls (Fig. 5 C). Moreover, even though the

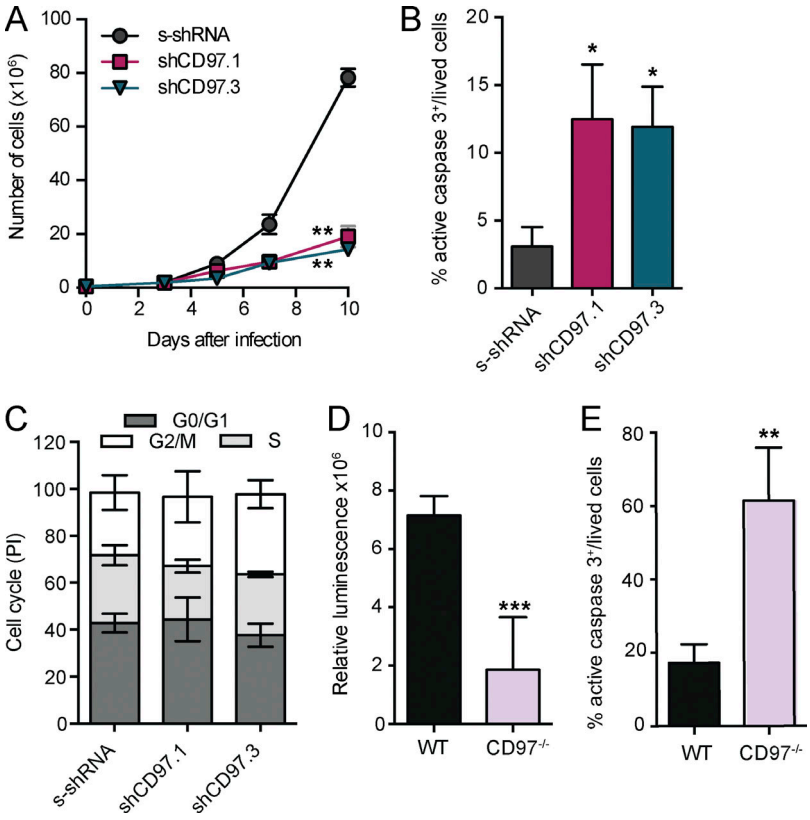


Figure 4. **CD97 promotes AML cell proliferation by increasing survival. (A)** Growth curves of CD97-KD or s-shRNA control HL60 cells measured by manual count. **(B)** Percentage of active caspase-3$^+$ cells in CD97-KD HL60 cells compared with s-shRNA control as assessed by flow cytometry. **(C)** Cell cycle analyzed in CD97-KD HL60 cells compared with s-shRNA control as assessed by flow cytometry. **(D)** Relative cell number of WT and CD97$^{-/-}$-MA9 cells in primary transplants as measured by CellTiter-Glo. **(E)** Percent active caspase-3$^+$ leukemic cells among WT and CD97$^{-/-}$-MA9 cells at the time of sacrifice. Data shown as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

AML cell line MOLM-13 is normally insensitive to differentiation upon ATRA treatment, CD97-KD alone was sufficient to induce expression of CD11b, CD14, and CD15, confirming the role of CD97 in maintaining the undifferentiated state of AML blasts (Fig. S4 D). To assess the specificity of the CD97-KD phenotype, we ectopically expressed CD97 in CD97-KD MOLM-13 cells using a lentivirus expressing human CD97 cDNA (CD97-OE). Confirming a role of CD97 in inhibiting myeloid differentiation, CD97 overexpression reduced expression of CD11b and CD14, largely rescuing the differentiation phenotype observed in CD97-KD cells (Fig. 5 D). These findings were confirmed in similar experiments ectopically expressing CD97 in MOLM-13 and OCI-AML3 cells, with CD97 overexpression resulting in decreased expression of CD15 and CD14 as well as increased expression of CD34 (Fig. S4 E). The overexpression of CD97 as well as the rescue of CD97 levels upon expression in CD97-KD cells were confirmed by flow cytometry (Fig. S4 F). We also performed similar KD experiments using primary human adult AML patient samples. Primary AML blasts transduced with CD97 shRNAs showed increased differentiation as demonstrated by increased expression of the differentiation markers CD11b and CD15 by flow cytometry (Fig. S4 G). Clinical information for all the patient samples have been listed in Fig. S4 H. These findings confirm the differentiation phenotype observed in cell lines following CD97-KD.

CD97 is dispensable for HSC function

As silencing of CD97 resulted in increased differentiation and decreased self-renewal of LSCs, inhibiting CD97 may be a potential therapeutic strategy in AML. To determine whether CD97-directed targeted therapies may affect normal hematopoiesis, we investigated the hematopoietic system of CD97$^{-/-}$ mice. Steady state complete blood counts revealed higher neutrophil and lymphocyte counts, similar to that previously described(Fig. S5 A). Total BM cell counts were comparable between CD97$^{-/-}$ and WT mice (Fig. S5 B), and no significant differences were detected in the frequency of BM HSCs or more committed progenitors; however, there was a mild increase in GMPs, consistent with the peripheral granulocytosis (Fig. S5 C).

To assess the function of CD97$^{-/-}$ HSCs, we FACS-sorted HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD48$^-$CD150$^+$) from CD97$^{-/-}$ and WT mice and performed methylcellulose colony formation (colony-forming cell [CFC]) assays. No differences in the number or types of colonies were observed between CD97$^{-/-}$ and WT cells after the first plating, but CD97$^{-/-}$ cells displayed increased colony-forming activity during the second round. In the third round of plating, CD97$^{-/-}$ cells showed a relative decrease in colony-forming activity, consistent with a loss in self-renewal (Fig. 6 A). We also measured the reconstitution potential of WT and CD97$^{-/-}$ HSCs in vivo by transplanting sorted HSCs into lethally irradiated congenic recipients. Transplantation of 1,000 CD97$^{-/-}$ HSCs resulted in a mild, but significant, increase in total CD45$^+$ donor chimerism in the BM 16 wk after transplantation (Fig. 6 B), but no differences were observed in granulocyteor lymphocyte engraftment. No differences were seen in the peripheral blood. This phenotype was associated with a higher frequency of immunophenotypically defined donor-derived HSCs in the BM of primary recipients (Fig. S5 D); however, differences in PB and BM total chimerism were not observed after the second transplantation (Fig. 6 C), even though there

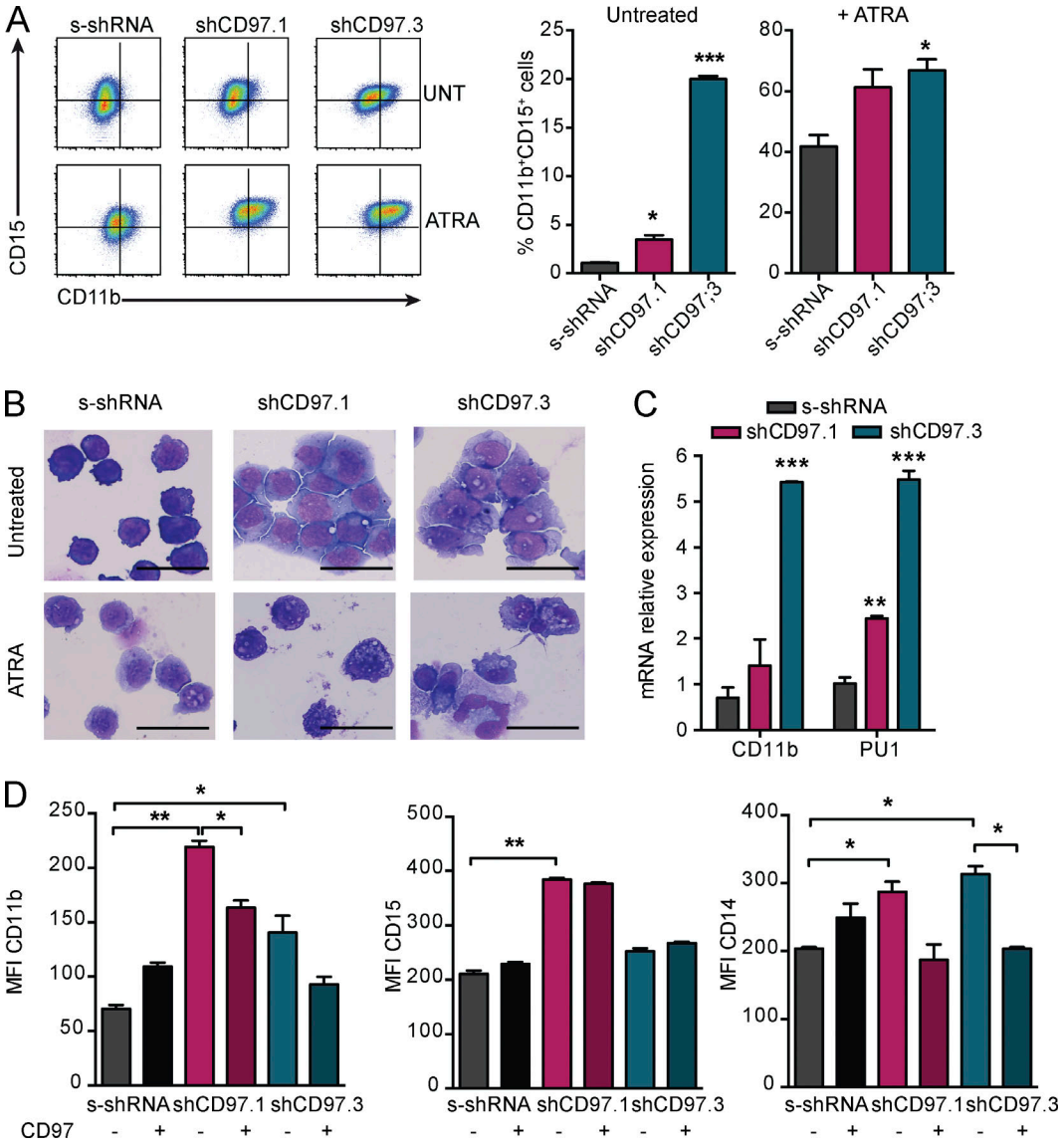


Figure 5. **Loss of CD97 induces AML cell differentiation. (A)** Representative dot plot and percentage of CD97-KD and control HL60 cells positive for the expression of myeloid lineage markers CD11b and CD15 after treatment with 10 nM ATRA for 4 d. **(B)** Wright-Giemsa stains of control and CD97-KD cells following ATRA treatment. Scale bar, 50 µm. (**C**) PU.1 and CD11b mRNA expression normalized to GAPDH, as measured by qPCR. **(D)** Myeloid differentiation marker expression on CD97-KD or control MOLM-13 cells expressing or lacking ectopic expression of CD97 cDNA. Data shown as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

was a mild decrease in myeloid progenitor chimerism in the BM after secondary transplant (Fig. S5 E).

Since CD97 is overexpressed in the vast majority of human AMLs, this suggests that it may play a direct role in AML pathogenesis. Thus, we investigated the effect of modulating CD97 expression on normal human HSPCs using CD97 shRNA and cDNA lentiviral vectors in CD34$^+$ umbilical CB cells. In CFC assays, CD97-KD CD34$^+$ cells gave rise to fewer colonies than s-shRNA infected cells, while CD97-OE induced increased colony formation (Fig. 6 D). These differences in colony formation were associated with impaired differentiation, with CD97-KD cells exhibiting significantly decreased CD34 expression, while CD97-OE cells showed increased CD34 expression compared with control cells (Fig. 6 E). To determine whether increased expression of CD97 blocks differentiation, we assessed myeloid differentiation in liquid culture in the presence of stem cell factor (SCF), IL-3, FLT3, and G-CSF (Egeland et al., 1991). Infection of CD34$^+$ cells with lentiviruses expressing CD97 induced a twofold increase in CD97 cell surface protein expression (Fig. 6 F). After 12 d in culture, CD97-OE cells expressed significantly lower levels of the myeloid markers CD11b, CD14, and CD15 (Fig. 6 G), consistent with partial blockade of HSPC maturation toward the myeloid lineage. Collectively, these data indicate that CD97 downregulation is necessary for normal myeloid differentiation, and provide additional evidence that one of the main functions of increased CD97 expression in human AML is to maintain the undifferentiated state.

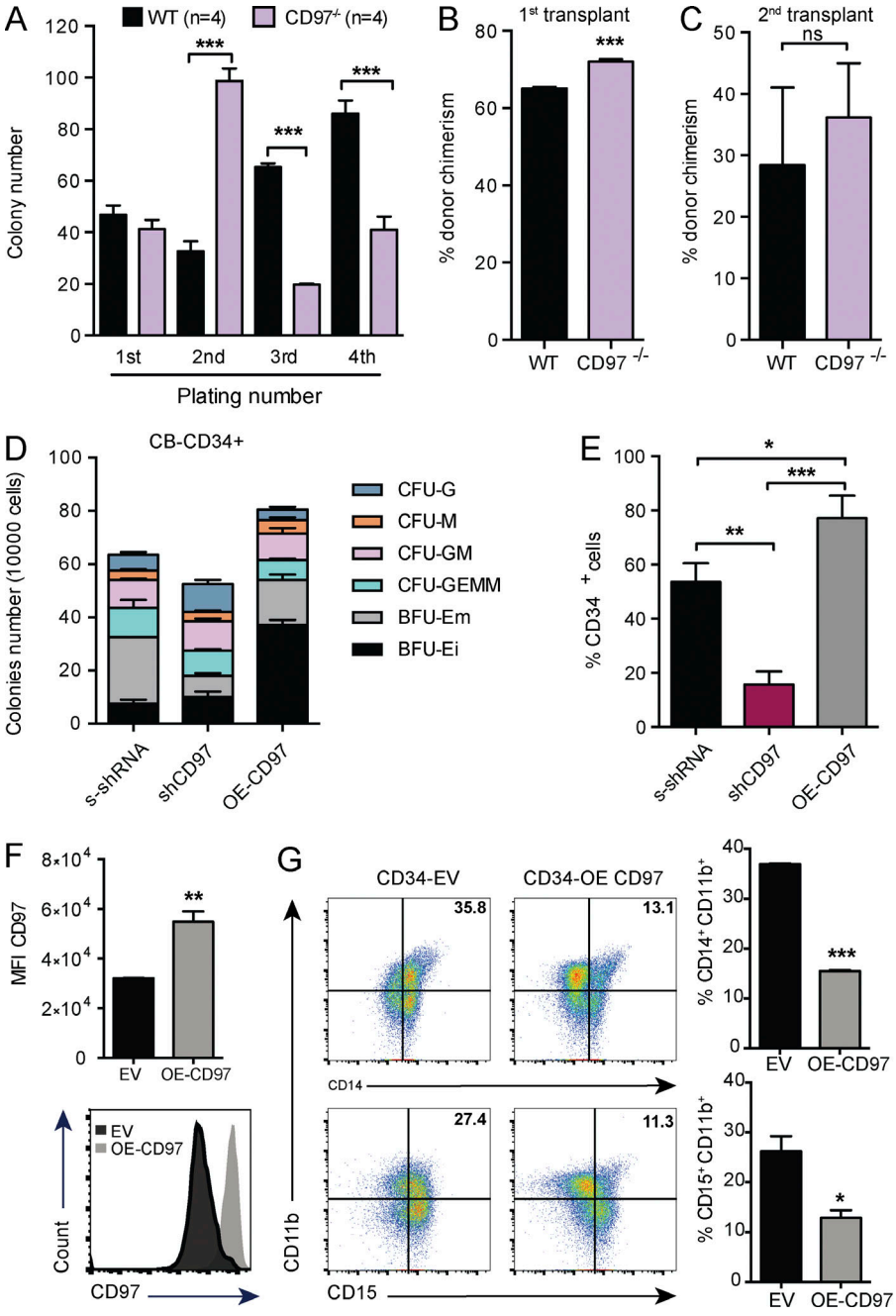


Figure 6. **CD97 down-regulation is required for normal myeloid cell differentiation. (A)** CFC numbers generated from FACS-sorted HSCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD150^+$) from WT or $CD97^{-/-}$ mice in both primary and serial replating experiments. **(B)** Lethally irradiated congenic mice were serially transplanted with HSCs from WT or $CD97^{-/-}$ mice. Donor chimerism in the BM was analyzed 16 wk after each transplantation ($n = 6$ per group). **(C)** Percentage of $CD45.2^+$ donor cells in BM were assessed 16 wk after secondary transplants. **(D)** Human $CD34^+$ enriched CB cells transduced with shCD97 or OE-CD97, and CFCs were assessed after 14 d. **(E)** Percentage of $CD34^+$ cells obtained after 14 d in methylcellulose culture (representative of two independent experiments). **(F)** CD97 expression measured by flow cytometry in $CD34^+$ CB cells OE-CD97 or control (EV). **(G)** $CD34^+$ CB cells infected with OE-CD97 or EV lentiviruses were grown in medium containing SCF, FLT3-L, IL-3, and G-CSF to induce myelomonocytic differentiation in vitro for 10 d. Representative dot plots showing expression of differentiation markers CD11b and CD15 ($n = 2$). Data shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ns, not significant between compared groups. BFU, blast-forming unit; Ei, immature erythroid; Em, mature erythroid; GEMM, granulocyte, erythrocyte, macrophage, megakaryocyte.

Discussion

AML arises from HSPCs and represents the end result of a multistep leukemogenic process that includes initiation, progression, and transformation. AML development requires the acquisition of cellular traits including increased resistance to apoptosis, increased self-renewal, and evasion of growth-suppressing signals from the microenvironment as well as the immune system, which enable replicative immortality of the cell (Weissman, 2005; Hanahan and Weinberg, 2011). These changes are due to alterations in both the genome and epigenome, leading to dysregulated expression of genes (Jan et al., 2012; Ley et al., 2013; Klco et al., 2014). These alterations are numerous and varied, as 50–60% of newly diagnosed AML patients harbor gross cytogenetic abnormalities (Martens and Stunnenberg, 2010), and numerous recurrent somatic mutations have been identified (Mardis et al., 2009; Patel et al., 2012). More recently, we and others have shown that AMLs are also characterized by significant epigenetic heterogeneity, even in the context of similar genetic alterations (Shih et al., 2015; Li et al., 2016). Together, these data have revealed that AML is a heterogeneous disease, with numerous mechanisms of transformation as well as potential differences in therapy responses and clinical outcomes (Ding et al., 2012; Walter et al., 2012). Despite this heterogeneity, novel treatment methods are being designed to target specific molecular pathways shown to have important roles in regulating leukemic cell growth, survival, and differentiation including DOT1L, IDH1/2, and others (Rau et al., 2016). However, it is becoming increasingly clear that single therapies

will likely be insufficient to achieve long-term remissions or cures AML patients. Developing therapies that target a wide range of AMLs, regardless of mutational status, and that target LSCs remains an important, if not largely unrealized goal.

While a number of cell surface molecules have been identified on AML blasts, few have been shown to regulate cell-intrinsic LSC function in primary leukemias. The best example of this is the TIM-3/Gal-9 autocrine stimulatory loop, which regulates self-renewal of human LSCs and promoted leukemic progression through coactivation of NF-κB and β-catenin signaling (Kikushige et al., 2015). In AML cell lines, IL1RAP decreases clonogenicity and increases cell death, but an LSC-supportive role in primary AML has not been explored (Barreyro et al., 2012). CD25, the receptor for IL-2, is required to maintain LICs in a murine model of CML, but this dependence has not been established in AML (Kobayashi et al., 2014). With respect to GPCRs, relatively little is known about their roles in AML biology. Recently, a role for GPR56 in AML has been described where high GPR56 expression is associated with high-risk genetic subgroups and poor outcome (Pabst et al., 2016). Thus, our studies represent a new description of the importance of the adhesion class of GPCRs in regulating LSC function.

In contrast to previously identified LSC antigens, our studies indicate that CD97 is more frequently overexpressed in AML patients and is expressed across different subtypes of AML, irrespective of their molecular, morphological, or histopathological subtypes. In addition, our functional studies revealed that CD97 supports the proliferation and survival of blasts in human cell lines and mouse models of AML. CD97-KD and CD97$^{-/-}$ leukemic cells consistently displayed growth reductions and a significant increase in apoptosis. CD97 also appears to play a major role in inhibiting myeloid differentiation, as demonstrated by the induction of differentiation via acquisition of markers of cellular maturation, changes in cell morphology and gene expression, and myelomonocytic maturation of leukemic cells upon CD97 loss. These findings were confirmed by the repression of cellular differentiation induced by overexpression of CD97. The effects of CD97 on AML differentiation are also seen in steady state hematopoiesis in CD97-deficient mice, which exhibit increased granulocytic differentiation and increased granulocyte precursor numbers. The role of CD97 in cellular differentiation was also confirmed in normal hematopoiesis, as CD97-KD in CD34^{+} CB cells reduced colony formation and increased GM differentiation, while CD97-OE resulted in increased colony formation and reduction in expression of cellular differentiation markers. Overall, these findings support a model in which CD97 is required in multiple steps during myeloid leukemogenesis: as a critical regulator of leukemic initiation, blast survival (i.e., disease progression), and LSC function (i.e., disease maintenance). CD97 overexpression likely regulates these processes predominantly through its ability to inhibit normal differentiation, associated with increased cell growth and decreased apoptosis, as well as an increase in self-renewal.

The relevance of CD97 to disease phenotypes was demonstrated using MA9 and AE9a murine models of AML, which showed dramatically reduced mortality and LSC function associated with increased differentiation and reduced self-renewal capacity in the context of CD97 loss. These effects on disease phenotypes were due to CD97's role in maintaining LSC function, as CD97-KD cells delayed mortality in NSG mice and CD97$^{-/-}$ LIC frequency was significantly reduced. This defect in leukemic maintenance does not appear to be due to defects in homing, as both WT-AE9a and CD97$^{-/-}$-AE9a blasts engrafted mice equally well up to 12 wk after transplantation, with exhaustion of CD97$^{-/-}$-AE9a blasts at 16 wk (Fig. S2 D). In addition, no difference in BM resident blasts was identified when equal numbers of WT-AE9a and CD97$^{-/-}$-AE9a blasts were transplanted into recipients (Fig. S2, F and G). Importantly, consistent with a critical role of CD97 in AML pathogenesis, AML patients expressing high levels of CD97 experienced worse clinical outcomes, with high CD97 mRNA expression predictive of poor OS in a multivariate Cox's proportional hazard model incorporating age, sex, blasts percentage, white blood cell count, cytogenetic subgroup, and somatic mutations. Notably, high levels of CD97 expression were associated with somatic mutations that are associated with both poor and favorable prognosis, including FLT3-ITD, NPM1, and RUNX1.

Several studies have demonstrated a central role for GPCRs such as GPR84, WNT, S1P, or CXCR4 in HSC maintenance (Doze and Perez, 2013; Lynch and Wang, 2016), and dysregulation of these pathways can have detrimental consequences, including malignant transformation. Because each GPCR is expected to stimulate multiple and highly interconnected cytoplasmic signaling pathways, largely through activation of AKT/MTOR, MAPKs, Hippo, or the Wnt/β-catenin signaling pathways, they can influence a wide array of biological responses (Laplante and Sabatini, 2012; Yu et al., 2012; O'Hayre et al., 2014). However, CD97 likely exerts its effects in a cell context–specific manner. For example, while GPCRs are generally thought to signal through Wnt/β-catenin, in colorectal carcinoma CD97 does not require canonical Wnt signaling (Wobus et al., 2006). In prostate and thyroid cancer models, CD97 heterodimerizes with the GPCR LPAR1 and mediates migration and invasion of cancer cells by increasing ERK1/2 phosphorylation and RHOA-GTP levels through activation of Gα12/13 (Ward et al., 2011, 2013). Thus, while a role for CD97 in different human solid cancers has been described, it is not clear if it acts through similar pathways or supports similar biological processes in AML.

As a member of the adhesion class of GPCRs, CD97 is thought to mediate its function by transducing signals from external cues, and a number of CD97 ligands have been identified, including chondroitin sulfate B (Stacey et al., 2003), α5β1 and αvβ3 integrins (Wang et al., 2005), CD90 (Wandel et al., 2012), and CD55 (Hamann et al., 1996; Kwakkenbos et al., 2005; Wang et al., 2005). While numerous studies have demonstrated higher expression of both CD97 and its ligand CD55 in primary gallbladder (Wu et al., 2012), pancreatic (He et al., 2015b), and gastric (Liu et al., 2012) carcinomas, as well as a correlation with metastasis, severity of the disease, and poor survival, none of these potential CD97–ligand interactions have been shown to be relevant in human cancer behavior. While we cannot exclude that CD97 may mediate interactions of AML blasts with previously defined or unknown external cues, our data support a significant

cell-intrinsic role for CD97, as loss of CD97 function by CD97 KD in AML cell lines ex vivo or by CD97 deletion in murine models of MA9 and AE9a AML strongly reduced cell proliferation and induced differentiation. Nonetheless, evaluation of CD97 signaling using transcriptomic and phosphoprotein array approaches in blasts with reduced or absent expression of CD97 revealed PI3K/Akt, STAT, and ERK/MEK pathways downstream of CD97. Thus, the role of CD97 in LSC proliferation and survival may be due to the activation of one or more of these pathways. Given that CD97 likely requires multiple signaling pathways to support its function, ultimately it is likely that inhibition of a single pathway will not be sufficient to inhibit all of CD97's functions, and thus therapeutic targeting of CD97 itself may provide a more robust alternative strategy to abrogate LSC function. Despite extensive in vitro and in vivo characterization, CD97$^{-/-}$ mice exhibited only minor alterations in peripheral blood counts and only mild alterations in HSPC function, which suggests that it may be possible to target leukemic cells with anti-CD97–directed therapies without significantly impacting normal hematopoiesis.

Materials and methods

Human primary AML samples

For validation of CD97 expression, 60 AML specimens representing 30 diagnostic:relapse pairs were analyzed. Human AML specimens were obtained from patients at Memorial Sloan Kettering Cancer Center, the University of Pennsylvania (Philadelphia, PA), the University of Adelaide (Adelaide, Australia), and the University of Rochester (Rochester, NY) with informed consent under institutional review board–approved protocols. CB was obtained from the New York Blood Center, and mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque Plus (Sigma-Aldrich). CD34^{+} cells were selected by magnetic separation using CD34 MicroBead Kit, human (Miltenyi Biotec).

Mice

The generation of CD97$^{-/-}$ mice has been described previously (Veninga et al., 2008). Donor and recipient mice (C57BL/6 and B6.SJL-Ptprca Pep/BoyJ, respectively) were bred and maintained in the Memorial Sloan Kettering Cancer Center animal facility. NSG mice were purchased from the Jackson Laboratory. All mice were maintained under pathogen-free conditions according to a protocol approved by the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee.

Cell culture

Human leukemia cell lines were cultured in RPMI 1640 supplemented with 10% FBS and 1× penicillin/streptomycin. Transformed mouse myeloid cells or leukemia cells were cultured in RPMI 1640 supplemented with 10% FBS, 10 ng/ml rmIL3, 50 ng/ml mouse stem cell factor, and 10 ng/ml rhIL6 (PeproTech) or in methylcellulose-containing medium (Methocult GF M3434; StemCell Technologies) with cytokines as previously described (Krivtsov et al., 2006). HEK293T and cells were maintained in DMEM supplemented with 10% FBS and 1× penicillin/streptomycin.

Real-time PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen) and converted to cDNA using SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative PCR (qPCR) was performed using an ABI StepOnePlus real-time PCR machine. Primers used for SYBR detection are available upon request. All signals were quantified using the ΔCt method and were normalized to GAPDH or β-actin mRNA expression levels.

Methylcellulose culture

Sorted cells were cultured in methylcellulose medium under myeloid conditions using Methocult M3434 or H3434 (StemCell Technologies). Cells were cultured in triplicate for 14 d. Colonies containing >50 cells were counted and classified under the microscope as previously described (Jordan et al., 2006).

shRNA design and virus generation

shRNAs (Table S1 and below) were designed using the RNAi Consortium shRNA library database (http://www.broadinstitute.org/rnai/public/) software to predict suitable target regions for each gene of interest. Sense and antisense oligonucleotides for each shRNA duplex were annealed and cloned into the lentiviral-based shRNA expression vector pRSI9-U6-(sh)-UbiC-TagRFP-2A-Puro: shCD97.1 target sequence, 5′-GCTGACCTATGTGTTTACCAT-3′; and shCD97.3 target sequence 5′-GCGATCCTTATGGCTCATTAT-3′.

Lentiviruses were generated by calcium phosphate–mediated cotransfection of shRNA constructs with pCMV-dR8.2 (packaging) and pCMV-VSVG (envelope) into HEK-293T cells, as previously described (Han et al., 2010). Supernatants were collected 48 and 72 h after transduction, and viruses were concentrated by ultracentrifugation (24,000 rpm, 4°C for 3 h). Retrovirus constructs MSCV-MLL-AF9-GFP and MSCVAML1-ETO9a-GFP and the packaging plasmid psi-Eco were used to produce retrovirus supernatants by cotransfection of HEK-293T cells as previously described (Krivtsov et al., 2006).

BM transduction and transplantation

CD97$^{-/-}$ and WT C57BL/6 mice were injected with 200 mg/kg 5-fluorouracil i.p. 6 d before sacrifice. Hematopoietic stem and progenitor cells were enriched from the BM by c-Kit magnetic bead selection (130-091-224; Miltenyi Biotec) and were cultured in the presence of recombinant murine cytokines, 50 ng/ml Flt3 ligand, 50 ng/ml SCF, 10 ng/ml IL-3, and 10 ng/ml IL-6 (PeproTech). 24 and 48 h after enrichment, c-Kit^{+} cells were infected with concentrated retrovirus supplemented with 6 µg/ml polybrene (Sigma-Aldrich) by spinoculation (1,450 rpm, 37°C for 1 h). Transduction efficiency was determined by reporter GFP fluorescence at 96 h by flow cytometry. For induction of AML, mice were sublethally (500 rad) or lethally (two rounds of 500 rad) irradiated and received varying doses of GFP^{+} transduced cells originating from WT or CD97$^{-/-}$ mouse donors together with 500,000 unfractionated BM cells for hemogenic support, by retroorbital injection. All the recipient hosts were sex and age matched.

Phosphoarray screen and Western blot analysis

For the phosphorylation-antibody array studies, cells were transduced with s-shRNA or CD97-KD shRNA, sorted 3 d later,

and used for antibody array analysis using the Kinex KAM-880 Antibody Microarray Kit (Kinexus), following the manufacturer's instructions. For Western blot analyses, cells were lysed in CelLytic buffer M (Sigma-Aldrich) supplemented with 1× Halt Protease and Phosphatase inhibitor cocktail (Thermo Fisher Scientific). Protein samples were loaded on NuPAGE (Invitrogen) 4–12% Bis-Tris gradient gels and transferred to nitrocellulose membranes (Invitrogen).

Flow cytometry and cell sorting

Mouse BM cells were harvested and stained as previously described. Briefly, antibodies used in this study include a lineage cocktail containing antibodies against Ter-119 (clone Ter-119), B220 (RA3-6B2), CD3e (145-2C11), CD4 (GK1.5), CD8 (53–6.7), Gr-1 (RB6-8C5), and Mac-1 (M1/70), conjugated with PE-Cy5 (eBiosciences). Additional antibodies used to identify HSPCs included CD16/32 (93) in Alexa eFluor 700, IL7Ra (A7R34) in PE-Cy5, CD45.1 (A20) in PE-Cy7, c-Kit (2B8) in allophycocyanin-eFluor 780, Gr-1 (RB6-8C5) in PE, CD45.2 (104) in Alexa eFluor 700, CD34 (RAM34) in fluorescein isothiocyanate, Flk2/Flt3 (A2F10) in PE (all from eBiosciences), as well as Sca-1 (E13-161.7) in Pacific Blue and CD150 (TC15-12F12.2) in PE (both from BioLegend). After staining, cells were analyzed and sorted using a FACSAria II (Becton Dickinson). All cell populations were double sorted, and a purity of >90% was routinely achieved. Mouse CD97 v2 Alexa Fluor 488–conjugated antibody (R&D Systems) and mouse anti-human CD97 allophycocyanin conjugate (clone MEM-180; Life Technologies) were used to stain mouse and human CD97, respectively. Flow cytometry data were analyzed using Flowjo software (TreeStar).

RNA-seq data analysis

For RNA-seq data generated from HL60 cells and MA9-transformed mouse cells, raw fastq files were aligned to the hg19 and mm10 reference genomes, respectively. Read counts were extracted using R with Rsamtools package (v1.24.0) and differentially expressed genes processed using the DESeq2 package (v 1.12.3). For normal human $CD34^+$ HSPC RNA-seq data, processed read count files were taken from the Human Reference Epigenome Mapping Project (sample GSM909310), and analysis of differential expression of genes between CD97 High and CD97 Low TCGA samples with normal $CD34^+$ cells was performed using DESeq2. ToppFun (Chen et al., 2009) and Panther (http://www.pantherdb.org) were used to identify enriched functional pathways. Heatmaps were generated using Morpheus (https://software.broadinstitute.org/morpheus). Data have been deposited in the Gene Expression Omnibus (accession no. GSE135028).

Survival analysis

155 of the 200 patients from the AML TCGA dataset (Ley et al., 2013) had corresponding CD97 expression values and had a French–American–British classification other than M3 (data annotations taken from cBioPortal, https://www.cbioportal.org/). Among these 155 patients, quartiles of expression values were calculated, and OS was estimated using Kaplan–Meier methods and compared across these four groups using a log-rank test. Cox proportional hazards regression estimated the univariate and multivariate associations with OS. Univariate associations with $P < 0.05$ were selected for the multivariate model. Individual mutations were included in the regression modeling if the mutation was observed in ≥10 individuals. To investigate the association of CD97 by cytogenetic risk, survival comparisons were conducted within each cytogenetic category. Due to sample size within each category, a median split of CD97 was used instead of quartiles. All survival analyses were done using the R statistical language (https://www.R-project.org/).

Statistical analysis

For statistical analyses, P values were determined by applying the two-tailed *t* test for independent samples. All values are expressed as means ± SEM, and *, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.001$. Analyses were performed with GraphPad Prism software. LIC frequency was estimated by linear regression analysis and Poisson statistics using publicly available ELDA software (Hu and Smyth, 2009).

Online supplemental material

Fig. S1 demonstrates the association of CD97 mRNA expression with different clinical correlates and patient outcomes in several AML datasets, as well as functional pathways associated with CD97 expression. It further demonstrates that shRNA-mediated KD of CD97 in the human AML cell line MOLM-13 results in longer survival in NSG mice engrafted with KD versus scramble control transduced cells. Fig. S2 shows that $CD97^{-/-}$c-Kit^+ MA9 and AE9a blasts exhibit poorer engraftment in mice compared with WT blasts. The differences in engraftment levels are not due to differences in homing ability, but rather differences in self-renewal, with $CD97^{-/-}$ blasts exhibiting increased differentiation. Fig. S3 shows RNA-seq evaluation of AML blasts in the context of CD97 KD, with CD97 KD in HL-60 cells and mouse MA9-transformed cells resulting in the positive enrichment of genes associated with cell death, differentiation, and proliferation and negative enrichment of genes associated with signal transduction, migration, and cytokine production. Fig. S4 demonstrates the importance of CD97 in maintaining the immature state of leukemic blasts and survival of blasts, as show in experiments using both human AML cell lines and primary adult AML patient samples. Fig. S5 shows that $CD97^{-/-}$ mice do not exhibit significant alterations in steady state hematopoiesis except for a mild granulocytosis. Table S1 lists multiple pathways associated with CD97 expression in CD97-KD HL60 versus CTRL s-shRNA infected cells, 5 d after infection, as determined by Kinexus antibody arrays. Dataset 1 shows all the GO biological processes differentially expressed in $CD97^{high}$ versus $CD97^{low}$ TCGA AML samples, shown in Fig. 1.

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