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Aberrant expression of the secreted protein, acidic, cysteine-rich (osteonectin) *SPARC* gene, which encodes a matricellular protein that participates in normal tissue remodeling, is associated with a variety of diseases including cancer, but the contribution of *SPARC* to malignant growth remains controversial. We previously reported that *SPARC* was among the most upregulated genes in cytogenetically normal acute myeloid leukemia (CN-AML) patients with gene-expression profiles predictive of unfavorable outcome, such as mutations in isocitrate dehydrogenase 2 (*IDH2*-R172) and overexpression of the oncogenes brain and acute leukemia, cytoplasmic (*BAALC*) and v-ets erythroblastosis virus E26 oncogene homolog (*ERG*). In contrast, *SPARC* was downregulated in CN-AML patients harboring mutations in nucleophosmin (*NPM1*) that are associated with favorable prognosis. Based on these observations, we hypothesized that *SPARC* expression is clinically relevant in AML. Here, we found that *SPARC* overexpression is associated with adverse outcome in CN-AML patients and promotes aggressive leukemia growth in murine models of AML. In leukemia cells, *SPARC* expression was mediated by the SP1/NF- $\kappa$ B transactivation complex. Furthermore, secreted *SPARC* activated the integrin-linked kinase/AKT (ILK/AKT) pathway, likely via integrin interaction, and subsequent  $\beta$ -catenin signaling, which is involved in leukemia cell self-renewal. Pharmacologic inhibition of the SP1/NF- $\kappa$ B complex resulted in *SPARC* downregulation and leukemia growth inhibition. Together, our data indicate that evaluation of *SPARC* expression has prognosticative value and *SPARC* is a potential therapeutic target [...]

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# SPARC promotes leukemic cell growth and predicts acute myeloid leukemia outcome

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<sup>1</sup>Division of Hematology, Department of Medicine, and <sup>2</sup>Comprehensive Cancer Center, The Ohio State University (OSU), Columbus, Ohio, USA.

<sup>3</sup>Alliance for Clinical Trials in Oncology Statistics and Data Center, Mayo Clinic, Rochester, Minnesota, USA. <sup>4</sup>Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA. <sup>5</sup>Department of Molecular Virology, Immunology and Cancer Genetics, and <sup>6</sup>Comparative Pathology and Mouse Phenotyping Shared Resource, Comprehensive Cancer Center, OSU, Columbus, Ohio, USA.

<sup>7</sup>Department of Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA.

**Aberrant expression of the secreted protein, acidic, cysteine-rich (osteonectin) (SPARC) gene, which encodes a matricellular protein that participates in normal tissue remodeling, is associated with a variety of diseases including cancer, but the contribution of SPARC to malignant growth remains controversial. We previously reported that SPARC was among the most upregulated genes in cytogenetically normal acute myeloid leukemia (CN-AML) patients with gene-expression profiles predictive of unfavorable outcome, such as mutations in isocitrate dehydrogenase 2 (IDH2-R172) and overexpression of the oncogenes brain and acute leukemia, cytoplasmic (BAALC) and v-ets erythroblastosis virus E26 oncogene homolog (ERG). In contrast, SPARC was downregulated in CN-AML patients harboring mutations in nucleophosmin (NPM1) that are associated with favorable prognosis. Based on these observations, we hypothesized that SPARC expression is clinically relevant in AML. Here, we found that SPARC overexpression is associated with adverse outcome in CN-AML patients and promotes aggressive leukemia growth in murine models of AML. In leukemia cells, SPARC expression was mediated by the SP1/NF-κB transactivation complex. Furthermore, secreted SPARC activated the integrin-linked kinase/AKT (ILK/AKT) pathway, likely via integrin interaction, and subsequent β-catenin signaling, which is involved in leukemia cell self-renewal. Pharmacologic inhibition of the SP1/NF-κB complex resulted in SPARC downregulation and leukemia growth inhibition. Together, our data indicate that evaluation of SPARC expression has prognosticative value and SPARC is a potential therapeutic target for AML.**

## Introduction

The secreted protein, acidic, cysteine-rich (SPARC) gene, located at chromosome bands 5q31–q33 (1), is highly conserved in vertebrates and encodes a 32-kDa matricellular protein also known as osteonectin or BM-40 (2, 3) that regulates cell-matrix interactions and tissue remodeling through the binding of collagen and other extracellular matrix proteins and activation of matrix metalloproteinases (2). SPARC also interacts with and regulates several growth factors, including TGF-β, FGF, VEGF, and PDGF (4–7). This pleiotropic activity suggests that the SPARC protein is likely to be expressed when tissue undergoes normal or pathologic remodeling.

SPARC expression has been shown to be altered in a variety of human conditions (e.g., diabetes, obesity) and in several types of cancer. However, the biologic and clinical significance of this gene is controversial, and conflicting reports have classified SPARC as either a tumor suppressor gene or an oncogene. The difficulty in assigning a specific function to the SPARC protein is related to the diverse roles that it can play both intracellularly in malig-

nant cells and extracellularly in the surrounding microenvironment (8). Low expression levels of SPARC were found in ovarian (9), colorectal (10, 11), and pancreatic cancer (12), whereas high SPARC expression was reported in breast cancer (13, 14), melanoma (15, 16), and glioblastoma (17). Stromal SPARC expression was associated with poor prognosis in non-small cell lung cancer (18) and with disease recurrence in breast ductal carcinoma *in situ* (19), whereas low stromal expression of SPARC predicted poor prognosis in colon cancer (20).

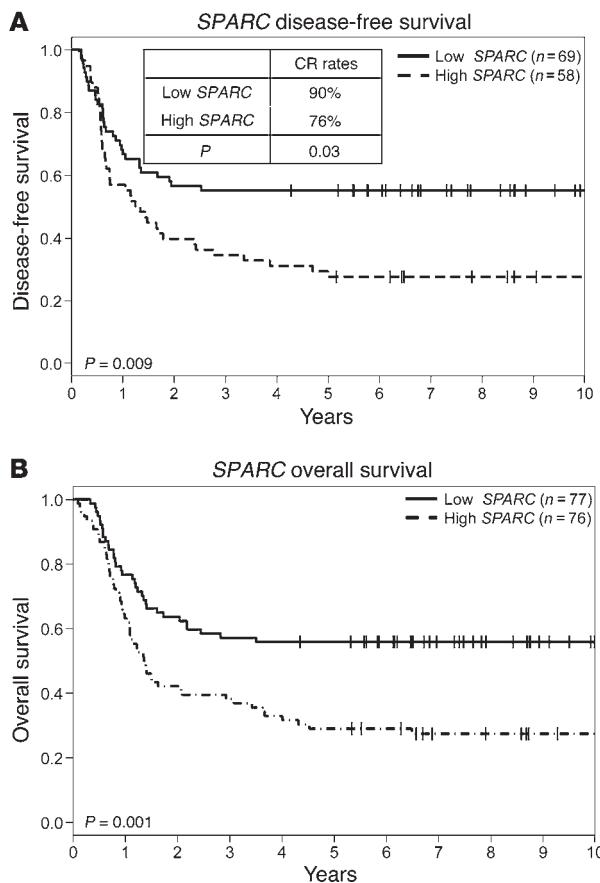
In hematologic malignancies, the role of SPARC is equally controversial. It was found to be downregulated at diagnosis in patients with del(5q) myelodysplastic syndromes (MDS) and upregulated following treatment with lenalidomide (21–23). SPARC was also found to be downregulated in acute myeloid leukemia (AML) with *MLL* rearrangements, usually associated with unfavorable prognosis, and upregulated in AML with *t(8;21)* or *inv* (16), which is usually associated with favorable prognosis, although no correlation of SPARC expression with outcome was reported (24). In chronic myelogenous leukemia, the accumulation of intracellular SPARC mediated by the Fyn/ERK signaling pathway seemingly contributed to imatinib resistance (25).

Recently, we observed that SPARC was upregulated in gene expression profiles (GEPs) associated with prognostically unfa-

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**Figure 1**

SPARC overexpression is associated with adverse clinical outcome in CN-AML. Outcome of CN-AML patients ( $n = 153$ ) according to *SPARC* expression levels. Median value of the measured *SPARC* expression levels was used to dichotomize patients into high and low expressers. (A) CR rates and DFS. (B) OS.

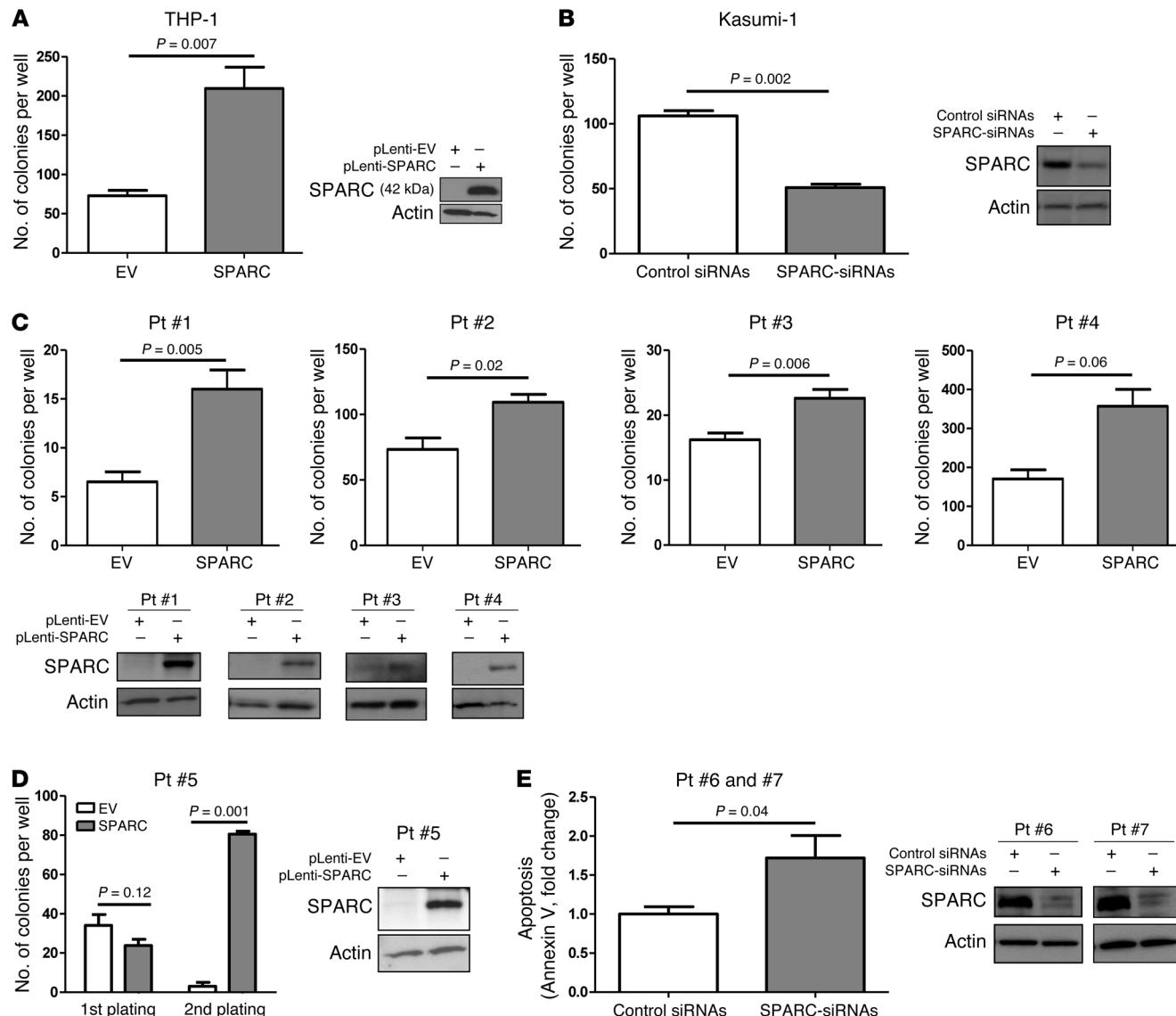
variable gene mutations (i.e., those in isocitrate dehydrogenase 2 [*IDH2*-R172]; ref. 26) or overexpressed genes (i.e., brain and acute leukemia, cytoplasmic [*BAALC*] and v-ets erythroblastosis virus E26 oncogene homolog [*ERG*]; ref. 27), and downregulated in GEP associated with the prognostically favorable nucleophosmin (*NPM1*) mutation (28) in cytogenetically normal AML (CN-AML). This led us to hypothesize that *SPARC* overexpression contributes to a more aggressive phenotype in AML. Thus, we dissected the clinical significance of *SPARC* overexpression in AML, the mechanisms by which this gene is deregulated, and the downstream effects of this aberrantly expressed gene. We show that *SPARC* overexpression independently predicts adverse outcome in CN-AML patients, thus representing what we believe to be a novel prognostic marker in AML. Consistent with these findings, we also demonstrate that *SPARC* contributes to leukemia growth in vitro and aggressive disease in vivo. *SPARC* overexpression activates integrin-linked kinase/AKT (ILK/AKT) and in turn  $\beta$ -catenin and could be targeted by modulating the SP1/NF- $\kappa$ B/*miR-29b* network, thereby also representing a potential therapeutic target in AML.

## Results

*SPARC* overexpression is associated with adverse clinical outcome in CN-AML. *SPARC* expression was analyzed by nCounter assays (NanoString Technologies) (29) in 153 younger (age range, 18–59 years) adults with primary CN-AML treated with cytarabine-daunorubicin-based regimens; clinical and molecular characteristics are shown in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI70921DS1).

Patients were dichotomized into higher and lower *SPARC* expressers using the median value cut-off. With a median follow-up of 8.7 years, higher *SPARC* expressers had lower odds of achieving a complete remission (CR) ( $P = 0.03$ ) and shorter disease-free survival (DFS) ( $P = 0.009$ ; 5-year DFS 28% vs. 55%) and overall survival (OS) ( $P = 0.001$ ; 5-year OS 29% vs. 56%) than lower expressers (Figure 1, A and B). In multivariable analyses, higher *SPARC* expression was independently associated with lower odds of CR ( $P = 0.007$ ), once adjusting for white blood count (WBC) ( $P = 0.003$ ), and shorter OS ( $P = 0.03$ ), once adjusting for *FLT3* internal tandem duplication (*FLT3*-ITD) ( $P < 0.001$ ), *WT1* ( $P = 0.003$ ), and *RUNX1* ( $P = 0.006$ ) mutations and WBC ( $P < 0.001$ ). There was also a trend for shorter DFS ( $P = 0.08$ ) once adjusting for *FLT3*-ITD ( $P < 0.001$ ) and *WT1* mutation ( $P = 0.004$ ). These data support the notion that *SPARC* is differentially expressed across AML patients and that the difference in the *SPARC* expression in this patient population has potentially biologic and clinical relevance.

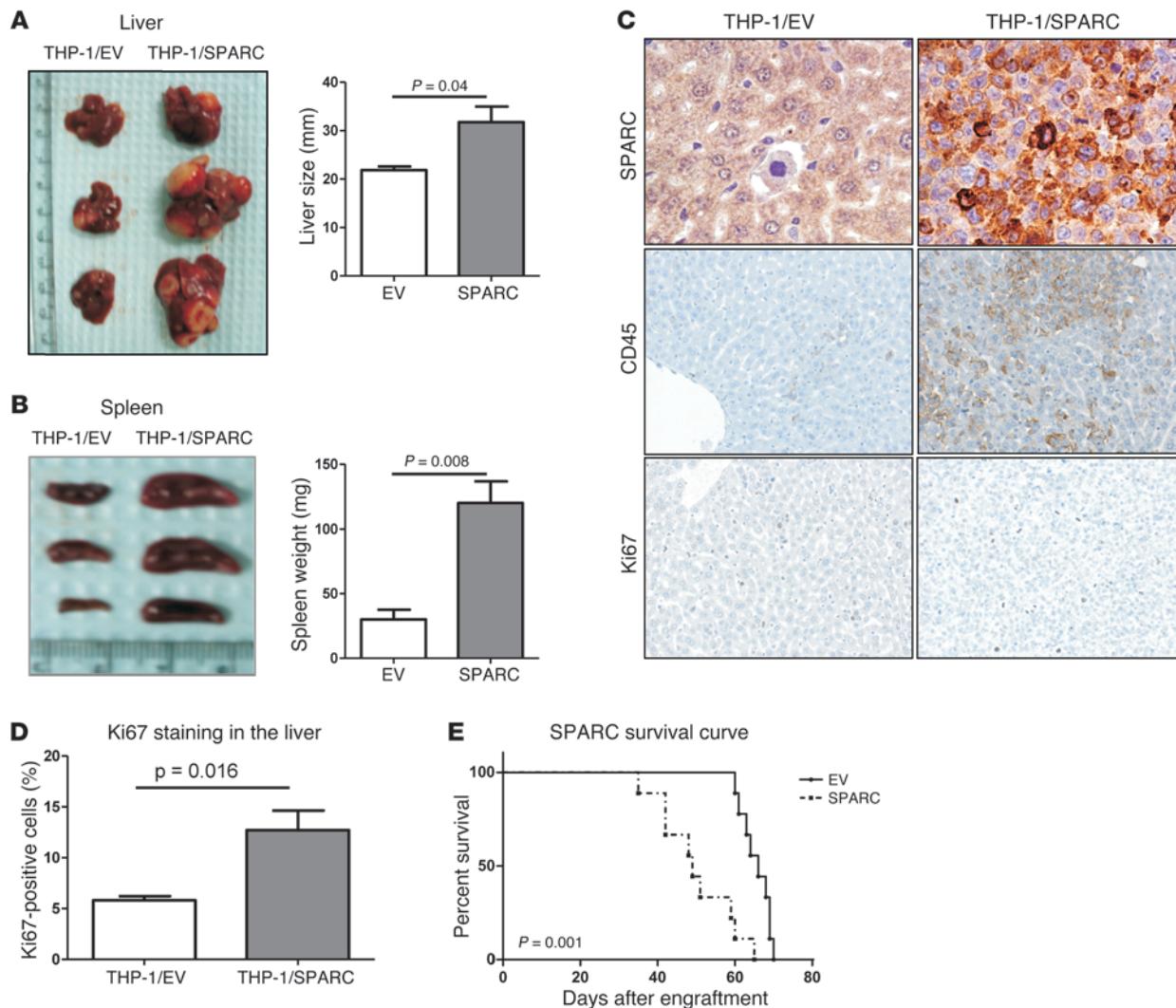
*SPARC* promotes leukemia growth in vitro and an aggressive disease in vivo. Having shown that higher *SPARC* expression was associated with adverse outcome, next we interrogated the *SPARC*-dependent mechanisms mediating the aggressive phenotype. THP-1 cells express a relatively lower endogenous level of *SPARC* mRNA (Supplemental Figure 1) and therefore represent a suitable in vitro model for gain-of-function experiments. THP-1 cells were stably infected with pLenti-*SPARC* (THP-1/*SPARC*) or empty vector as a control (THP-1/EV). Ectopic expression of *SPARC* was confirmed by Western blotting (Figure 2A). A significant increase in the ability of colony formation was observed in THP-1/*SPARC* cells compared with the THP-1/EV cells (mean, 209 vs. 72;  $P = 0.007$ ; Figure 2A). Likewise, when we knocked down *SPARC* (Figure 2B) in Kasumi-1 cells that express a relatively higher endogenous levels of the gene (Supplemental Figure 1), we observed a decrease in the colony-forming ability in *SPARC* siRNA-transfected cells compared with the control siRNA-transfected cells (mean, 106 vs. 50;  $P = 0.002$ ; Figure 2B). Similar experiments were also conducted in primary blasts from 5 AML patients (numbers 1–5) expressing a relatively lower level of endogenous *SPARC* and 2 patients (numbers 6, 7) expressing a relatively higher level of endogenous *SPARC* (see patients' cytogenetic and molecular features in Supplemental Table 2). For patients 1–5; blasts were infected with either the pLenti-*SPARC* or the pLenti-EV vector. A significant increase in colony forming ability was observed in *SPARC*-infected blasts compared with EV controls for patients 1–4 (means, 16 vs. 7;  $P = 0.005$ ; 109 vs. 73;  $P = 0.02$ ; 23 vs. 16;  $P = 0.006$  and 357 vs. 170;  $P = 0.06$ ) (Figure 2C). In blasts from a patient 5, ectopic *SPARC* expression did not significantly increase the colony-forming ability after first plating, but a significant increase in the number of colonies was observed after the secondary plating (mean, 80 vs. 3;  $P = 0.001$ ; Figure 2D). For patients 6 and 7, we observed an increase in spontaneous apoptosis in AML blasts when *SPARC* endogenous expression was knocked down by siRNAs (Figure 2E), suggesting that *SPARC* promotes both growth and survival of leukemia cells.


**Figure 2**

SPARC promotes growth advantage in AML. **(A)** SPARC protein levels (right panel) and quantification of colony formation (left panel) in THP-1 cells ectopically expressing SPARC. **(B)** SPARC knockdown (right panel) and quantification of colony formation (left panel) in Kasumi-1 cells. **(C)** SPARC ectopic expression in primary blasts from 4 AML patients (numbers 1–4; bottom panel) and quantification of colony formation assays in these cells (top panel). **(D)** Western blotting showing SPARC protein levels (right panel) and quantification of colony formation in primary blasts from AML patient 5 ectopically expressing SPARC scored at 14 days following first and second plating (left panel). **(E)** SPARC protein levels measured in primary blasts from 2 AML patients (numbers 6–7) transfected with SPARC siRNA (right panel) and quantification of apoptosis determined by annexin V staining in SPARC-siRNAs versus control siRNA–transfected blasts (left panel). Data represent mean  $\pm$  SEM.

To examine the contribution of SPARC to leukemia growth *in vivo*, NOD/SCID/gamma (NGS) mice were injected with THP-1/SPARC cells or THP-1/EV cells via tail vein. Eight weeks later, 3 mice in each group were sacrificed. Bone marrow engraftment of the leukemia cells was confirmed by CD45 antibody staining. The THP-1/SPARC mice had significantly larger livers ( $P = 0.04$ ; Figure 3A) and spleens ( $P = 0.008$ ; Figure 3B) than THP-1/EV controls. THP-1/SPARC cells formed coalescing masses obliterating large expanses of the liver parenchyma (Supplemental Figure 2), while THP-1/EV cells were observed either alone or in small clusters within hepatic sinusoids without effacing the hepatic cords.

The THP-1/SPARC cells had a monomorphic phenotype and a seemingly higher proliferative capacity compared with the more pleomorphic character and increased apoptotic tendency of the THP-1/EV cells (see immunohistochemistry (IHC) staining for SPARC, CD45, and Ki67 in Figure 3C and quantification of Ki67 in Figure 3D). Histopathology of spleen sections showed increased infiltration of leukemic cells and destruction of splenic architecture in tissues obtained from THP-1/SPARC mice compared with tissues obtained from THP-1/EV mice (Supplemental Figure 2). The THP-1/SPARC mice ( $n = 9$ ) also had a significantly shorter survival than the THP-1/EV mice ( $n = 9$ ) ( $2 \times 10^6$  cells were injected

**Figure 3**

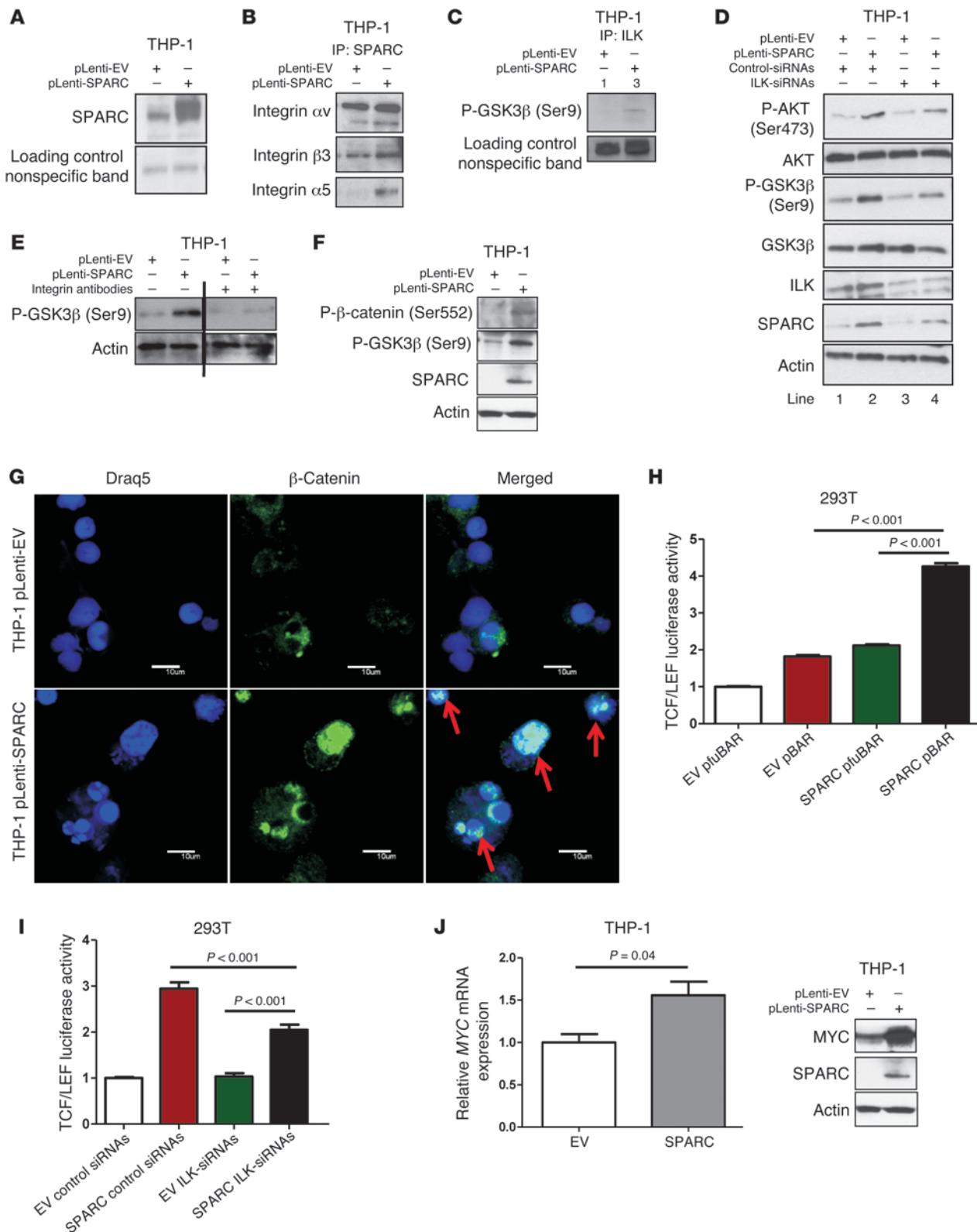
SPARC promotes aggressive disease in vivo. NSG mice were engrafted with THP-1 cells infected with pLenti-EV or pLenti-SPARC vectors; mice were sacrificed and tissues were collected 8 weeks following engraftment. (A) Liver sizes (mean  $\pm$  SEM). (B) Spleen sizes (mean  $\pm$  SEM). (C) Immunohistochemical staining for SPARC, CD45, and Ki67 expression in liver tissues obtained from THP-1/EV and THP-1/SPARC mice. Original magnification,  $\times 400$ . (D) Quantitative analysis for Ki67 (proliferation marker) expression in liver tissues obtained from THP-1/EV and THP-1/SPARC mice (using ImmunoRatio Software). (E) Survival analysis of THP-1/SPARC mice ( $n = 9$ ) compared with THP-1/EV controls ( $n = 9$ ).

per mouse; median survival, 49 vs. 66 days;  $P = 0.001$ ; Figure 3E). Similar results were obtained in a repeated experiment where a higher number of cells ( $5 \times 10^6$ ) were injected into each mouse (Supplemental Figure 3). Consistent with these results, mice injected with the SPARC-shRNA-infected CG-SH cells, which otherwise express high levels of endogenous SPARC, showed a trend for smaller spleens than mice injected with control shRNA-infected cells (Supplemental Figure 4).

**SPARC activates ILK/AKT/β-catenin signaling pathways.** Next, we asked how SPARC contributes to the mechanisms of leukemia growth. Several cell-surface receptors have been identified as interacting with the secreted SPARC protein, including some of the membrane integrins (30, 31). This interaction activates ILK, a multifunctional cytoplasmic serine/threonine kinase (32–34) that phosphorylates and activates AKT and phosphorylates and

inactivates glycogen synthase kinase 3β (GSK3β; Ser9). GSK3β promotes β-catenin degradation, but once phosphorylated, its activity is suppressed, and β-catenin stabilizes and translocates into the nucleus (35). Activated AKT phosphorylates and stabilizes β-catenin at residue Ser552 (36). Thus, the net result of the SPARC/integrin/ILK interplay is enhanced β-catenin. The latter is required for self-renewal of leukemia stem cells (LSCs) (37–41). Thus, we hypothesized that SPARC overexpression leads to aberrant β-catenin activation in AML.

SPARC is a secreted protein (2, 3). We found significantly higher levels of SPARC in the media collected from THP-1/SPARC cell cultures than in the media collected from THP-1/EV cell cultures (Figure 4A). We incubated THP-1 cells with conditioned media from THP-1/SPARC cells and THP-1/EV cells and performed colony assay 48 hours after incubation. We observed higher num-



**Figure 4**

SPARC activates ILK/AKT/β-catenin signaling pathways in THP-1 cells and enhances β-catenin nuclear translocation and transcriptional activity. THP-1 cells were transfected with pLenti-EV or pLenti-SPARC vectors. (A) Western blot measuring secreted SPARC protein levels in media collected from cell culture of THP-1 cells infected with pLenti-SPARC vector compared with cells infected with pLenti-EV. (B) SPARC and integrin coimmunoprecipitation assay. (C) ILK kinase activity assay. Lysates from THP-1/SPARC cells and THP-1/EV cells were immunoprecipitated with anti-ILK antibody and assayed utilizing GSK3 fusion protein (ILK substrate). Numbers represent ratio of band intensity to control band. (D) Western blot analysis of P-AKT(Ser473) and P-GSK3β(Ser9) levels in THP-1/SPARC cells compared with THP-1/EV in the presence of endogenous ILK or following ILK knockdown or (E) following blocking integrin receptors with integrin antibodies. Lanes separated by a line were run on the same gel but were noncontiguous. (F) Western blot analysis of P-β-catenin (Ser552) in THP-1/SPARC cells compared with THP-1/EV cells. (G) Immunofluorescence confocal microscopy images showing β-catenin (green) and cell nuclei (blue) in EV- or SPARC-transfected THP-1 cells stained with β-catenin antibody and Drag5 antibody (nuclear staining); arrows indicate cells exhibiting β-catenin nuclear translocation. Scale bars: 10 μm. (H) Luciferase activity measured in 293T cells following cotransfection with pBAR (TCF/LEF reporter vector) or pfuBAR (TCF/LEF reporter vector with mutated site) and pLenti-EV or pLenti-SPARC and (I) in the presence of control siRNAs or ILK siRNAs. (J) *MYC* mRNA expression (left panel) and protein expression (right panel) in THP-1/SPARC cells compared with THP-1/EV. Data represent mean ± SEM for 3 different experiments.

ber of colonies in THP-1/SPARC media incubated THP-1 cells compared with THP-1/EV media incubated cells (Supplemental Figure 5), suggesting that the conditioned media from SPARC transfected cells induce cell growth in SPARC-nonexpressing cells. Coimmunoprecipitation assays with SPARC and surface integrins showed binding of the protein at least to integrins α<sub>v</sub>, β<sub>3</sub>, and α<sub>5</sub> (Figure 4B) and lower or no binding to integrins β<sub>1</sub>, α<sub>4</sub>, and β<sub>4</sub> (data not shown). Kinase activity assay of anti-ILK immunoprecipitate from THP-1/SPARC cells incubated with recombinant GSK3 showed increased P-GSK3β(Ser9) (mean 2.4 ± 0.68 of 3 different experiments) as compared with the immunoprecipitate from THP-1/EV cells, thereby reflecting higher ILK activity in the presence of SPARC (Figure 4C). Furthermore, increased endogenous P-AKT(Ser473) and P-GSK3β(Ser9) were detected in THP-1/SPARC cells compared with THP-1/EV cells (Figure 4D, lines 1 and 2). Knocking down ILK (Figure 4D, lines 3 and 4) decreased AKT and GSK3β phosphorylation in THP-1/SPARC cells. Consistently, functional inhibition of ILK activity using the T315 (ILK inhibitor) (42) abolished SPARC-induced cell growth as assessed by MTS and colony-forming assay (Supplemental Figure 6).

ILK activates AKT through PI3K activation (43), suggesting that pharmacologic blockade of PI3K signaling should result in abrogating the SPARC effect on AKT and GSK3β phosphorylation. Indeed, THP-1/SPARC cells treated with 50 nM PI3K inhibitor LY294002 showed decreased AKT(Ser473) and P-GSK3β(Ser9) compared with untreated THP-1/SPARC cells (Supplemental Figure 7).

Next, we tested whether blocking integrin receptors would decrease SPARC function. GSK3β phosphorylation in THP-1/SPARC cells was reduced in THP-1/SPARC cells incubated with antibodies against α<sub>v</sub>, β<sub>3</sub>, and β<sub>1</sub> (Figure 4E); indeed, blocking α<sub>v</sub>β<sub>3</sub> was sufficient to decrease SPARC-dependent GSK3β phosphorylation (Supplemental Figure 8). Consistent with these findings, we observed higher levels of P-β-catenin(Ser552) (Figure 4F) and an

increased β-catenin nuclear translocation (Figure 4G) in THP-1/SPARC compared with THP-1/EV cells.

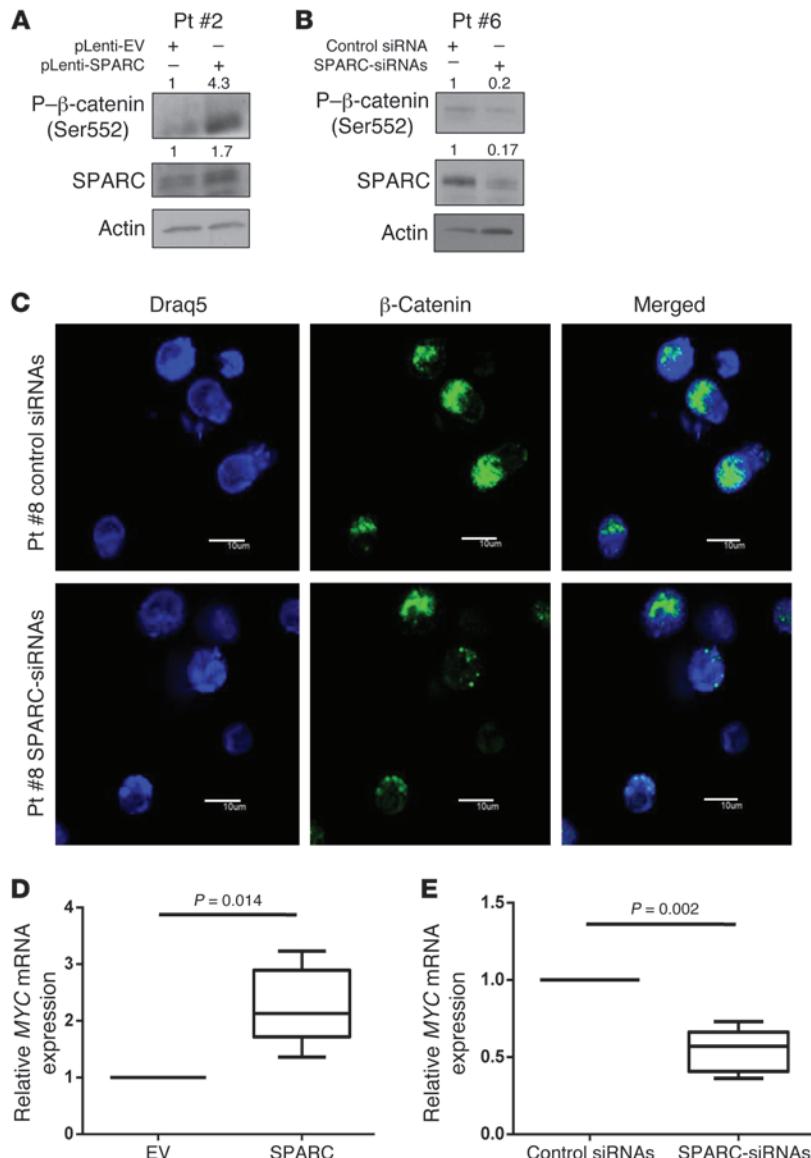
Once in the nucleus, β-catenin interacts with TCF/LEF transcription factors and promotes expression of genes supporting cell growth and proliferation (e.g., *MYC* and *CCND1*). Ectopic SPARC expression resulted in an approximately 4-fold increase in TCF/LEF reporter activity, as measured by luciferase assay in SPARC-transfected 293T cells compared with EV-transfected controls ( $P < 0.001$ ; Figure 4H). The SPARC-induced TCF/LEF activity decreased upon ILK siRNA knockdown (Figure 4I). SPARC protein levels in 293T cells assessed by Western blot are shown in Supplemental Figure 9. Increase in SPARC-induced β-catenin-TCF/LEF activity was also supported by upregulation of the TCF/LEF target gene *MYC* (Figure 4J). We also observed consistent results in other cell lines (e.g., MV4-11 cells) forced to express SPARC (Supplemental Figure 10).

Finally, we validated our findings in blasts from AML patients. Forced expression of SPARC increased P-β-catenin (Ser552) (Figure 5A; patient 2), while siRNA SPARC knockdown decreased P-β-catenin(Ser552) (Figure 5B; patient 6) and total β-catenin (Figure 5C; patient 8). SPARC overexpression and knockdown in primary blasts resulted, respectively, in significant increase and decrease of *MYC* mRNA expression (Figure 5, D and E). To ensure that increased TCF/LEF target gene expression was not due to other leukemogenic mechanisms operative in AML cells, we also showed that forced SPARC could increase *MYC* and *CCND1* expression in CD34<sup>+</sup> cells from cord blood cells of normal donors (Supplemental Figure 11).

It has been reported that β-catenin is expressed and activated in LSCs and that it is required for self-renewal (37, 38). Thus, we postulated that SPARC expression may be higher in less differentiated hematopoietic cell subpopulations. To prove this, we showed that SPARC expression was significantly higher in the CD34<sup>+</sup> compared with CD34<sup>-</sup> cells in normal bone marrow cells (NBM) ( $n = 4$ ;  $P < 0.001$ ; Figure 6A), and in CD34<sup>+</sup>/CD38<sup>-</sup> HSCs/multipotent progenitors (HSC/MPP) compared with CD34<sup>+</sup>/CD38<sup>+</sup> cells (common-myeloid progenitors [CMP] and granulocyte-monocyte progenitors [GMP]) both in NBM ( $n = 3$ ) and AML blasts ( $n = 3$ ; patients n. 1,5 and 9) (Figure 6, B and C). Furthermore, we showed that SPARC-transduced primary AML blasts (patient 9) indeed exhibited a higher percentage of engraftment in NSG mice than those transduced with GFP/EV control (3.1% vs 0.4% circulating blasts 12 weeks after blast i.v. injection; Figure 6D).

*SP1, NF-κB, and miR-29b modulate SPARC expression in AML.* Next, we asked which mechanisms lead to aberrant SPARC expression in AML. We identified binding sites for SP1, NF-κB, and RUNX1 in the (-651/1) promoter region (Transcriptional Regulatory Element Database [TRED] website; <http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home>) and binding sites for *miR-29* in the 3' UTR region of the human *SPARC* gene. Indeed, *SPARC* has been reported to be a *miR-29* target (44). Notably, we have recently shown that activation and overexpression of oncogenes involved in AML (i.e., *KIT*, *FLT3*, and *DNMTs*) require interaction and transactivation of the transcription factors SP1 and NF-κB and inhibition of *miR-29b* (45). Therefore, we postulated that a similar mechanism could be responsible for SPARC overexpression.

To test this hypothesis, we first performed luciferase gene reporter assays by cloning the *SPARC* promoter region spanning the SP1-, NF-κB-, and RUNX1-binding sites into the pGL4.11 luciferase vector. Then, 293T cells were cotransfected with the reporter gene and vectors expressing SP1 and p65 (NF-κB subunit). A signifi-


**Figure 5**

SPARC activates ILK/AKT/β-catenin signaling pathways in primary blasts. **(A)** P-β-catenin (Ser552) in primary blasts (AML patient 2) ectopically expressing SPARC and **(B)** in primary blasts (AML patient 6) transfected with SPARC siRNAs. Numbers represent ratio of band intensity to the control band. **(C)** Immunofluorescence confocal microscopy images showing β-catenin (green) and cell nuclei (blue), respectively, in primary blasts (AML patient 8) stained with β-catenin antibody and Draq5 antibody (nuclear staining) following SPARC knockdown. Scale bars: 10 μm. **(D)** Box plots showing MYC mRNA expression measured in primary blasts transfected with pLenti-SPARC compared with those transfected with pLenti-EV ( $n = 3$ ). **(E)** Box plots showing MYC mRNA expression measured in primary blasts transfected with SPARC siRNAs compared with those transfected with control-siRNAs ( $n = 4$ ). The boxes correspond to the 25th and 75th percentiles, the whiskers correspond to the minimum to maximum values, and the horizontal lines indicate the median.

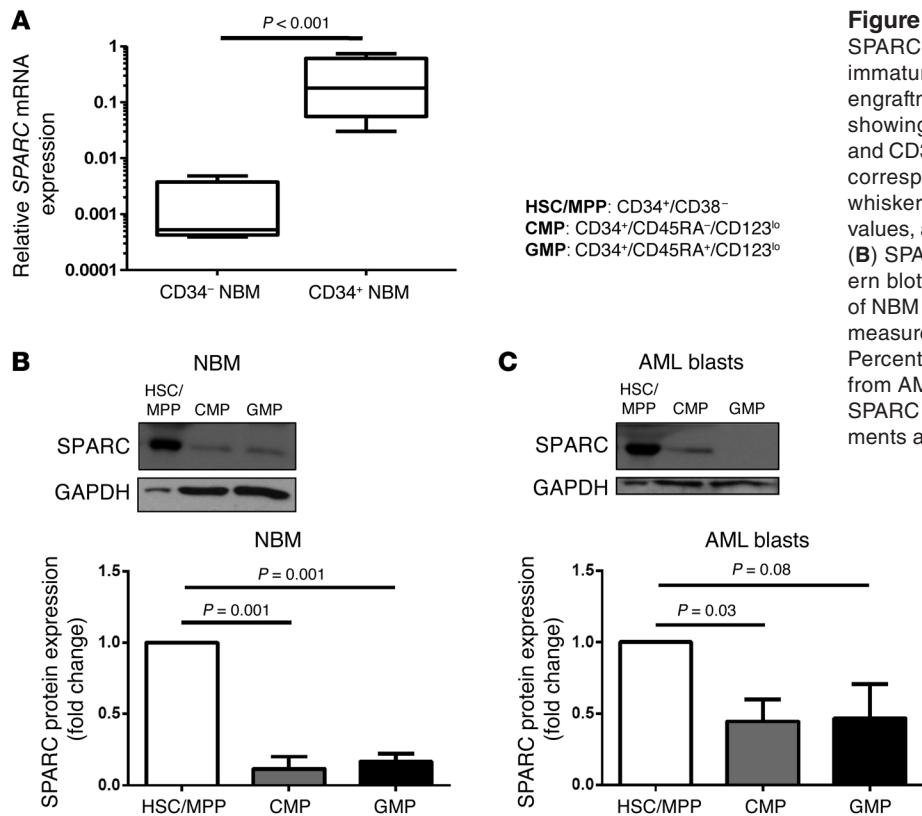
cant increase in luciferase activity was found in cells transfected with *SP1*- or *p65*-expressing vectors compared with EV controls ( $P < 0.001$  for both; Figure 7A). Ectopic expression of *SP1* and *p65* also resulted in significant increases in endogenous *SPARC* expression in THP-1 cells (Figure 7, B and C), whereas *SP1* and *p65* siRNA knockdown resulted in significant decrease of *SPARC* expression in Kasumi-1 cells (Figure 7, D and E).

A significant decrease of *SPARC* promoter luciferase activity was found in 293T cells transfected with *RUNX1* expression vector ( $P < 0.001$ ; Figure 7F). In contrast, *RUNX1*-WT knockdown resulted in *SPARC* upregulation in MV4-11 cells (Figure 7G), while knockdown of *RUNX1*-*RUNX1T1* translocation by siRNAs resulted in a significant *SPARC* downregulation in *RUNX1*-*RUNX1T1*-positive Kasumi-1 cells (Figure 7H).

Notably, ectopic expression of *p65* and *SP1* increased their respective enrichment on the *SPARC* promoter (Figure 7, I and J), and forced *RUNX1*-WT expression decreased *SP1*, but not *p65*, enrichment as measured by ChIP in THP-1 cells (Figure 7K).

These results are also consistent with the finding that *RUNX1*-mutated CN-AML patients exhibit higher *SPARC* expression when compared with the *RUNX1*-WT patients (Supplemental Figure 12). Together, these results indicate that *SP1*, *p65* and *RUNX1* loss-of-function promoted *SPARC* expression, while *RUNX1*-WT inhibited *SPARC* expression.

As *SPARC* was found to be a target for *miR-29a* and *miR-29c* (44) and the *SPARC* 3' UTR region was predicted to have binding sites for *miR-29b* (46, 47), we cloned the *SPARC* 3' UTR region in luciferase reporter vector (pGL3) and cotransfected this construct with the synthetic microRNA (*miR-29b* and *miR-9\**) or scramble oligos in 293T cells. We found a 60% decrease of *SPARC* 3' UTR-luciferase activity in cells cotransfected with *miR-29b* compared with the activity in cells cotransfected with scramble ( $P < 0.001$ ) or an unrelated microRNA: *miR-9\** (Figure 8A). Kasumi-1 cells transfected with *miR-29b* showed significant decreases in endogenous *SPARC* (Figure 8B). AML blasts transfected with *miR-29b* also showed significant decreases in *SPARC* levels at 24 hours (Figure 8C).

**Figure 6**

SPARC expression is higher in normal and AML immature cell subpopulations and enhances blast engraftment in immunodeficient mice. (A) Box plot showing SPARC mRNA level measured in CD34<sup>+</sup> and CD34<sup>-</sup> cells of NBM samples ( $n = 4$ ). The boxes correspond to the 25th and 75th percentiles, the whiskers correspond to the minimum to maximum values, and the horizontal lines indicate the median. (B) SPARC protein expression measured by Western blot in sorted stem cell/progenitor populations of NBM samples ( $n = 3$ ) and (C) AML blasts ( $n = 3$ ); measurements are presented by mean  $\pm$  SEM. (D) Percentage of engraftment of primary AML blasts from AML patient 9 transduced with either MIGR-SPARC or MIGR-EV in NSG mice ( $n = 2$ ); measurements are presented by mean  $\pm$  SEM.

We have previously shown that *miR-29b* is downregulated in AML via a SP1/NF- $\kappa$ B/HDAC inhibitory complex and that pharmacologic disruption of this complex results in *miR-29b* upregulation and downregulation of direct (i.e., SP1, DNMT3A, DNMT3B) and indirect (i.e., DNMT1, FLT3, KIT) *miR-29b* targets (45, 48, 49). Consistent with these reports, we showed that bortezomib at concentrations achievable *in vivo* (45, 50) significantly increased *miR-29b* expression (Figure 8D) and in turn decreased *SP1* and *SPARC* mRNA and protein levels in Kasumi-1 cells (Figure 8, E and F) and in blasts from 3 different patients with primary AML (numbers 6, 10, and 7) (Figure 8, G and H). Consistent with the *in vitro* results, we found *SPARC* expression was significantly reduced in blood and spleen from CG-SH cell-engrafted NSG mice treated with bortezomib (1 mg/kg) twice a week for 4 weeks compared with vehicle-treated mice (Supplemental Figure 13).

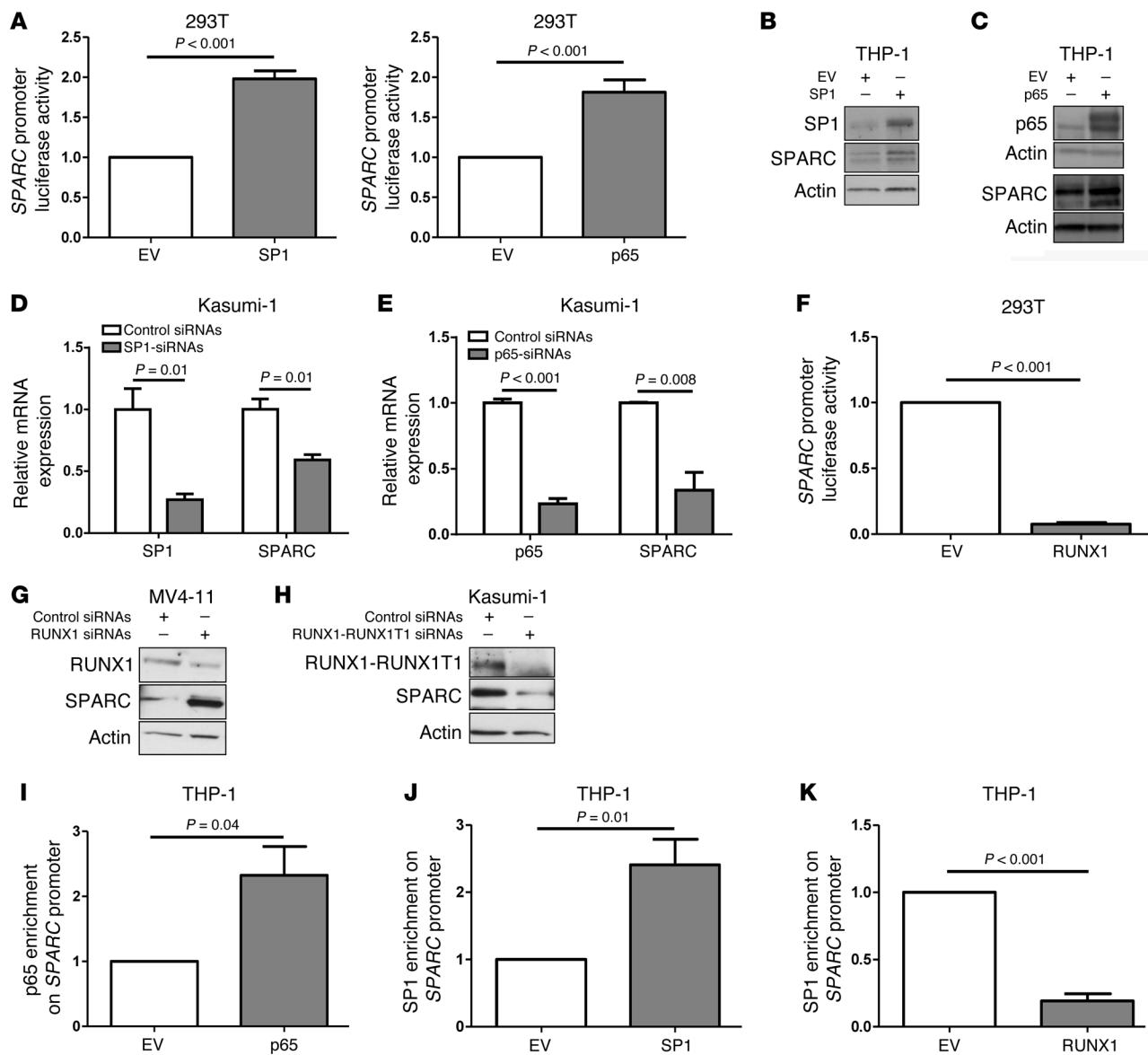
## Discussion

Although altered *SPARC* expression has been observed in cancer, the clinical significance of both upregulation and downregulation of the gene and the encoded protein remain to be fully elucidated (15, 17, 51–58). In contrast with solid tumors, the number of reports investigating this gene in hematologic malignancies is relatively small. In MDS, 5q deletion results in allelic loss and significant downregulation of *SPARC* expression in the progenitor compartment (22, 23), and a dramatic upregulation of *SPARC* occurs in response to lenalidomide (23). Although this suggests that *SPARC* expression is a treatment-response predictor and perhaps plays a role in normal hematopoiesis, to date, no biological evidence supporting this notion has been reported. Indeed, *SPARC* was found to be dispensable for murine hematopoiesis (59). Nev-

ertheless, the complexity of the hematopoietic role of *SPARC* is illustrated by a recent report (60). These authors showed in a myeloproliferative murine model that increased *SPARC* expression in the bone marrow stroma favored fibrotic changes, while loss of *SPARC* expression resulted in a defective stromal niche (60).

Here, we sought to dissect the role of *SPARC* in AML, starting from the observation that this gene was found overexpressed in GEPs associated with distinct molecular subsets of patients harboring prognostically unfavorable mutations or overexpressed genes. Supporting the hypothesis that *SPARC* overexpression likely contributes to aggressive myeloid leukemogenesis, we showed that high *SPARC* expression was associated with adverse outcomes in CN-AML patients. Although *SPARC* overexpression was associated with other unfavorable molecular markers at diagnosis, in multivariable models for outcome, the impact of *SPARC* remained significant even after adjusting for other molecular prognosticators, supporting an independent role of this gene in determining an aggressive clinical phenotype. To our knowledge, this is the first study reporting the clinical relevance of *SPARC* expression in AML. We validated these findings through mechanistic experiments using AML preclinical models and primary blasts. We showed that *SPARC* upregulation promoted an aggressive phenotype in AML cells. Mice engrafted with AML cells forced to express *SPARC* had increased leukemia burden and shorter survival compared with controls.

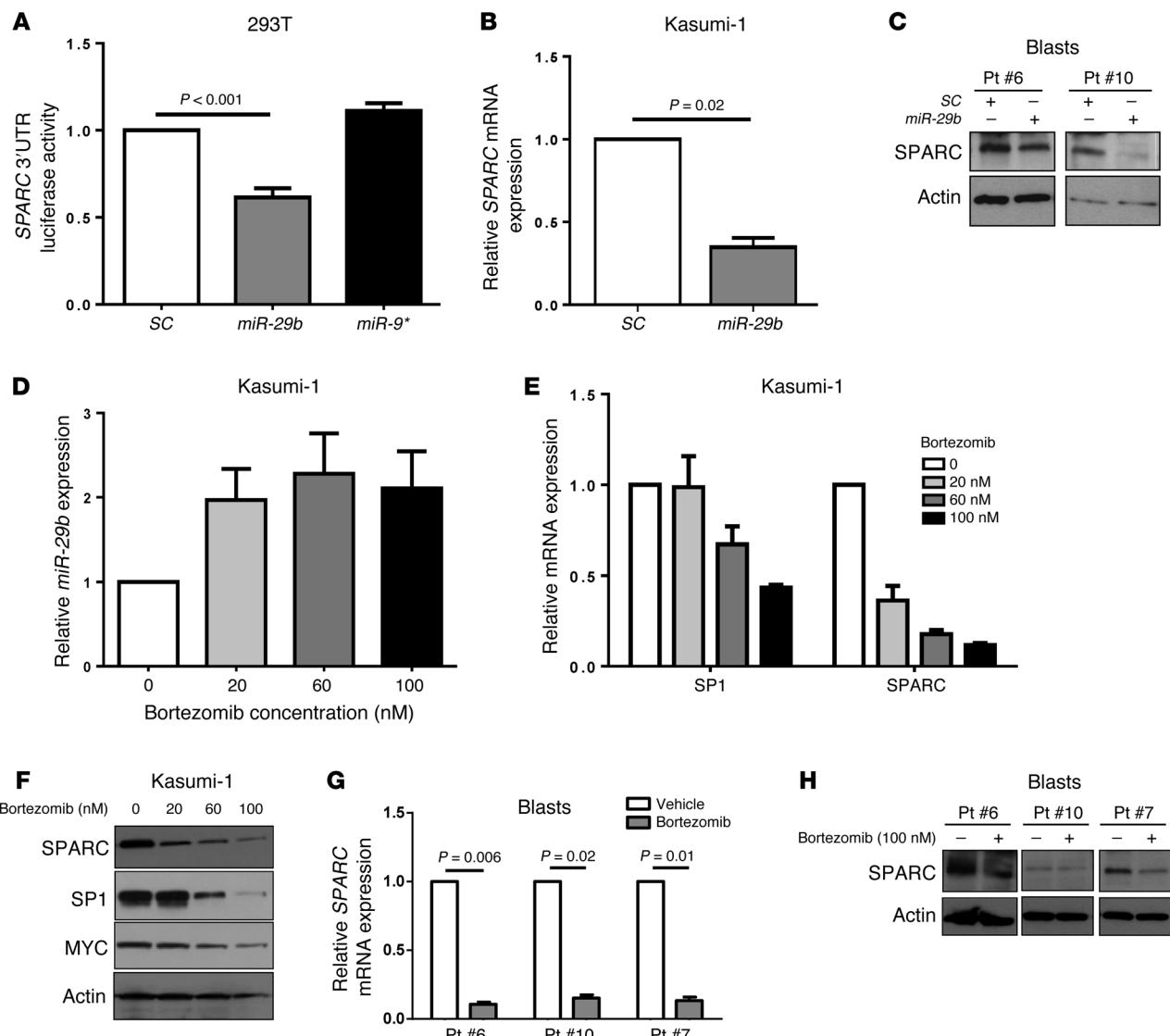
The mechanisms leading to *SPARC* upregulation in AML blasts are multifactorial and likely converge to NF- $\kappa$ B-dependent pathways, which have been previously shown to be constitutively activated in AML LSCs (61). We found that *SPARC* may function through an autocrine mechanism; once secreted by AML cells,

**Figure 7**

SP1, NF-κB, and *miR-29b* modulate SPARC expression in AML. (A) Luciferase activities measured in 293T cells cotransfected with PGL4.11 luciferase vector containing the SPARC promoter region (PGL4.11/SPARC promoter) and either SP1-expressing (left panel) or p65-expressing (right panel) vectors; measurements were normalized to that obtained in 293T cells cotransfected with PGL4.11/SPARC-promoter vector or EV. (B) Western blot analysis for SPARC protein expression measured in THP-1 cells transfected with SP1-expressing vector or (C) p65-expressing vector. (D) SPARC mRNA expression measured in Kasumi-1 cells transfected with SP1 siRNAs or (E) p65 siRNAs. (F) Luciferase activities measured in 293T cells cotransfected with a PGL4.11 luciferase vector containing the SPARC promoter region (PGL4.11/SPARC-promoter) and a RUNX1-expressing vector; measurements were normalized to that in 293T cells cotransfected with PGL4.11/SPARC-promoter vector and EV. (G) Western blot analysis showing SPARC protein levels in MV4-11 cells transfected with RUNX1 siRNAs or (H) Kasumi-1 cells transfected with RUNX1-RUNX1T1 siRNA. (I) ChIP showed p65 enrichment on SPARC promoter in THP-1 cells transfected with p65-expressing vector. (J) ChIP showed SP1 enrichment on SPARC promoter in THP-1 cells transfected with SP1-expressing vector. (K) Change in SP1 enrichment on SPARC promoter in RUNX1-transfected THP-1 cells compared with EV-transfected cells. Data represent mean  $\pm$  SEM.

the protein interacted with the leukemia cell membrane integrins and activated ILK/AKT/β-catenin signaling, thereby promoting cell engraftment, growth, and survival. Pharmacologic disruption of the SP1/NF-κB transactivation complex (45, 62) resulted in SPARC downregulation, thereby representing a potentially novel therapeutic strategy for SPARC-dependent AML.

A finding in our study was that SPARC enhanced β-catenin activity in AML cells, known to be required for leukemia growth and stemness. The increase in β-catenin activity was seemingly enhanced via the SPARC-integrin-ILK axis. ILK is a kinase that links the cell adhesion receptors, integrins, and growth factors with the downstream signaling pathways involving AKT and

**Figure 8**

Targeting SPARC expression in AML. **(A)** SPARC 3' UTR–luciferase activity in the presence of *miR-29b* was measured by cotransfeting 293T cells with SPARC-3' UTR reporter and a synthetic microRNA; values were normalized to those of cells transfected with scramble. **(B)** Ectopic expression of synthetic *miR-29b* significantly decreases SPARC mRNA expression in Kasumi-1 cells. **(C)** SPARC protein in AML blast samples (patients 6 and 10) at 24 hours following transfection with *miR-29b*. **(D)** *miR-29b* levels, **(E)** mRNA levels of SPARC and SP1, and **(F)** Western blot analysis showing SPARC, SP1, and MYC protein levels, measured in Kasumi-1 cells treated with 20, 60, and 100 nM of bortezomib. **(G)** SPARC mRNA and **(H)** protein levels in blasts from 3 primary AML samples (patients 6, 10, and 7) treated with 100 nM bortezomib and assessed 24 hours later.

GSK3 $\beta$  (43). This ultimately results in stabilization and nuclear localization of  $\beta$ -catenin and activation of genes promoting cell-cycle progression and proliferation (63). Consistent with these results, we observed that SPARC levels were higher in both normal and immature AML cell subpopulations that indeed displayed a higher engraftment potential in immunodeficient mice. While SPARC/ILK/AKT signaling was found involved in glioma cell invasion and survival (64), to our knowledge, the relevance of this mechanism in AML has not been previously reported.

Having observed that SPARC overexpression contributes to more aggressive phenotype in AML patients, we asked whether it is possible to target SPARC expression and demonstrate the clinical relevance of our findings. We showed that, similarly to other

oncogenes, constitutive SPARC expression was dependent on a previously reported regulatory molecular network that involves SP1/NF- $\kappa$ B and *miR-29b* (45). The leukemogenic role of this network that can be targeted pharmacologically has been extensively studied by our group not only in AML (45) but also in other types of leukemia (45, 62). Our data support the view that SPARC overexpression is a potentially actionable therapeutic target via pharmacological disruption of the SP1/NF- $\kappa$ B/*miR-29b* network by, for example, bortezomib, used here as a proof of concept. Other compounds, including inhibitors of HDAC (e.g., vorinostat, AR-42) or SP1 (e.g., mithramycin A) or synthetic *miR-29b* mimics may also interfere with the SP1/NF- $\kappa$ B/*miR-29b* network and downregulate SPARC (45, 50, 65, 66).



While our findings emphasize a role of SPARC in the homeostasis of myeloblasts, several other reports have underscored the interplay of SPARC protein with the microenvironment to create a niche favorable for cancer growth (8). Thus, future studies of SPARC in AML need to address the dual intracellular and extracellular role of this protein.

In conclusion, *SPARC* deregulation is clinically relevant in AML because overexpression of this gene independently predicts adverse outcome in subsets of AML patients, contributes to aggressive AML growth likely via  $\beta$ -catenin activation, and is an actionable therapeutic target. This suggests that future clinical studies targeting the activity of SPARC protein are warranted in AML.

## Methods

**Plasmids and reagents.** SPARC clone was purchased from Invitrogen and inserted in pLenti6.2/V5-DEST (Invitrogen) and MIGR (Addgene). pCMV-p65, pSUPER-SP1, and pCMV-RUNX1 expression vectors were also used (45). OFF-TARGET control and ON-TARGET plus siRNA-SMARTpool reagents against SPARC, SP1, p65, and RUNX1 (each contains a mix of at least 4 different sequences) were purchased from Dharmacon. siRNA against RUNX1-RUNX1T1 was custom designed (Dharmacon). SPARC shRNA (a set of 6 different sequences) was purchased from Thermo Fisher Scientific. Bortezomib is commercially available (Millennium Pharmaceuticals).

**Cell lines and primary blasts.** THP-1, MV4-11, Kasumi-1, and CG-SH cells were cultured in RPMI medium supplemented with 10%–20% FBS. Blasts from AML patients were maintained in RPMI medium supplemented with 20% FBS and 1 $\times$  StemSpan CC100 (StemCell Technologies). AML blasts used in the experiments were obtained from apheresis blood samples collected from patients treated at OSU and stored in the OSU Leukemia Tissue Bank.

**Transient transfection, RNA interference, and viral induction.** Transient transfection of cells was performed utilizing 1 to 2  $\mu$ g of plasmid or 1 nmol of siRNA per reaction and Nucleofector Kit (Amaxa) according to the manufacturer's instructions (67). Retroviral and lentiviral infection were performed with an MOI of 3 and greater than 10, respectively, to obtain efficiencies that approximately reached 40%, as previously reported (68, 69). Cells were selected by antibiotic selection with blasticidin or sorting for GFP-positive cells.

**RNA extraction and RNA expression quantification.** Total RNA was extracted using Trizol reagent (Invitrogen). SPARC expression in CN-AML patients was measured by Nano-String nCounter system according to the manufacturer's instructions (Nano-String). Gene cDNA was synthesized using SuperScript III reagents (Invitrogen) and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time RT-PCR (qRT-PCR) was performed using commercially available TaqMan Gene Expression Assay primers and probes and the 7900HT Fast Real-Time PCR System (Applied Biosystems). The expression levels were normalized to 18S for gene or U44 for microRNA expression.

**Immunoprecipitation, Western blot analysis, ChIP, and antibodies.** Immunoprecipitation and Western blot were performed as previously described (70). ChIP assays were performed using the EZ ChIP Kit (Millipore) according to the manufacturer's protocol. DNA was quantified using qRT-PCR with SYBR green incorporation (Applied Biosystems) and primers specific for SPARC promoter and normalized to their input. Antibodies and primers are listed in Supplemental Experimental Procedures.

**Clonogenic and viability analyses.** Methylcellulose clonogenic assays were carried out by plating 1  $\times$  10<sup>3</sup> cells of different cell lines or 2  $\times$  10<sup>4</sup> primary blasts in 0.9% MethoCult (StemCell Technologies) (68). Colony numbers were scored 10 days later.

For viability and apoptosis analysis, cells were washed with PBS and resuspended in 50  $\mu$ l binding buffer containing 2  $\mu$ l of annexin V (eBioscience)

and 5  $\mu$ l propidium iodide (PI) (eBioscience). After 20 minutes incubation, fluorescence was quantified by flow cytometry on a FACSCalibur instrument.

**Immunofluorescent staining, cell sorting, flow cytometry and confocal microscopy.** Cells were washed and stained with CD34, CD38, CD45RA, and CD123 antibodies (eBioscience) and sorted into hematopoietic stem cells and HSC/MPP (CD34<sup>+</sup>/CD38<sup>-</sup>), CMP (CD34<sup>+</sup>/CD45RA<sup>-</sup>/CD123<sup>lo</sup>) and GMP (CD34<sup>+</sup>/CD45RA<sup>+</sup>/CD123<sup>lo</sup>) using BD FACS Aria II cell sorter (BD Biosciences).

For confocal microscopy analysis, 1  $\times$  10<sup>6</sup> cells were processed as detailed in Supplemental Experimental Procedures.

**In vivo experiments.** Four- to six-week-old NOD/SCID mice (The Jackson Laboratory) were i.v. injected via tail vein with 5  $\times$  10<sup>6</sup> THP-1 cells infected with plenti-EV or plenti-SPARC. Eight weeks later, mice ( $n$  = 6) were euthanized; spleens, livers, and sternums were isolated. Xenograft transplants were performed as detailed in Supplemental Experimental Procedures.

**Patients.** We studied pretreatment bone marrow and blood samples with 20% or more blasts from 153 patients with primary CN-AML (age range, 18–59 years) who were treated with cytarabine-daunorubicin-based regimens on Cancer and Leukemia Group B (CALGB) 9621 or 19808 protocols (71). Per protocol, no patient included in our analysis received allogeneic transplantation in first CR. The median follow-up was 8.7 years.

**Cytogenetic and mutational analyses.** The diagnosis of CN-AML was based on the analysis of 20 or more metaphases in pretreatment bone marrow specimens subjected to short-term cultures and confirmed by central karyotype review (72). Patients were also characterized centrally for *FLT3*-ITD (73), *FLT3* tyrosine kinase domain (TKD) mutations (74), *MLL* partial tandem duplication (75, 76), *NPM1* (28, 77), *WT1* (78), *CEBPA* (79), *IDH1*, and *IDH2* (26) *TET2* (80), *ASXL1* (81), and *DNMT3A* (71) mutations as previously reported.

**Statistics.** Statistical analyses relative to clinical outcome were performed by the Alliance Statistics and Data Center. Definitions of clinical end points – CR, DFS, and OS – are as reported previously (26). The differences in baseline clinical and molecular features between higher and lower *SPARC* expressers were tested using the Fisher's exact and Wilcoxon rank sum tests for categorical and continuous variables, respectively. Estimated probabilities of DFS and OS were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions. Mechanistic and biological experiments were analyzed with paired and unpaired 2-tailed *t* tests as required, and a *P* value of less than 0.05 was considered significant. Data are presented as mean  $\pm$  SEM in all figures where error bars are shown.

**Study approval.** Informed consent to use patient samples for investigational studies was obtained from each patient according to OSU institutional guidelines and in accordance with the Declaration of Helsinki, and protocols were approved by the IRB at OSU and each center involved in the CALGB trials. All animal studies were performed in accordance with OSU institutional guidelines for animal care and under protocols approved by the OSU Institutional Animal Care and Use Committee.

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Address correspondence to: Guido Marcucci, The Ohio State University Comprehensive Cancer Center, Biomedical Research Tower, 460 W. 12th Ave, Columbus, Ohio 43210, USA. Phone: 614.366.2261; Fax: 614.688.4028; E-mail: [guido.marcucci@osumc.edu](mailto:guido.marcucci@osumc.edu).



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