

ORIGINAL ARTICLE

Low or absent *SPARC* expression in acute myeloid leukemia with *MLL* rearrangements is associated with sensitivity to growth inhibition by exogenous *SPARC* proteinJF DiMartino¹, NJ Lacayo^{2,3}, M Varadi¹, L Li¹, C Saraiya^{2,3}, Y Ravindranath⁴, R Yu^{2,3}, BI Sikic^{2,3}, SC Raimondi⁵ and GV Dahl^{2,3}

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Secreted protein, acidic and rich in cysteine (SPARC), is a matricellular glycoprotein with growth-inhibitory and antiangiogenic functions. Although SPARC has been implicated as a tumor suppressor in humans, its function in normal or malignant hematopoiesis has not previously been studied. We found that the leukemic cells of AML patients with *MLL* gene rearrangements express low to undetectable amounts of SPARC whereas normal hematopoietic progenitors and most AML patients express this gene. SPARC RNA and protein levels were also low or undetectable in AML cell lines with *MLL* translocations. Consistent with its tumor suppressive effects in various solid tumor models, exogenous SPARC protein selectively reduced the growth of cell lines with *MLL* rearrangements by inhibiting cell cycle progression from G1 to S phase. The lack of *SPARC* expression in *MLL*-rearranged cell lines was associated with dense promoter methylation. However, we found no evidence of methylation-based silencing of *SPARC* in primary patient samples. Our results suggest that low or absent *SPARC* expression is a consistent feature of AML cells with *MLL* rearrangements and that SPARC may function as a tumor suppressor in this subset of patients. A potential role of exogenous SPARC in the therapy of *MLL*-rearranged AML warrants further investigation.

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Introduction

Rearrangements involving the *Mixed Lineage Leukemia (MLL)* gene on chromosome 11q23 are found in 5–10% of acute myeloid leukemia (AML) patients and in some studies confer an increased risk of treatment failure.¹ The role of the bone marrow microenvironment in mediating resistance to therapy has not been extensively studied. In particular, aberrant interactions between leukemia cells and the extracellular matrix (ECM) could potentially play a role in the pathogenesis of *MLL*-rearranged AML. The bone marrow microenvironment provides important signals to hematopoietic progenitors that affect their growth, survival and differentiation through regulated contacts with stromal cells and the ECM.² A prime example of the importance of these interactions is the recently described hematopoietic stem cell (HSC) niche, in which direct interaction with osteoblasts provides growth and survival signals for HSC.^{3,4} A role for cell–matrix interaction in this niche is suggested by the

observation that osteopontin, a matricellular glycoprotein produced by osteoblasts, can modulate the proliferation of HSC.^{5,6}

Matricellular glycoproteins are a structurally unrelated family of proteins that includes, in addition to osteopontin, thrombospondins 1 and 2, tenascins C and X and secreted protein, acidic and rich in cysteine (SPARC) (osteonectin, BM-40). Altered expression of any of these proteins could potentially contribute to neoplasia by perturbing cell–matrix interactions. SPARC in particular has been frequently implicated in human cancer.⁷ This 40 kDa glycoprotein mediates diverse functions including inhibition of cell proliferation through its effects on collagen and modulation of cell signaling by transforming growth factor beta (TGF- β) and proangiogenic cytokines.^{8,9} Loss of *SPARC* expression has been reported in pancreatic cancer and addition of exogenous SPARC or its enforced expression reduces the growth and tumorigenicity of pancreatic, ovarian and breast cancer cells.^{10–13} *SPARC* expression by adjacent normal stroma also exerts anti-tumor effects. In neuroblastoma, for example, production of SPARC by stromal Schwann cells inhibits tumor growth *in vivo* by an antiangiogenic mechanism.¹⁴ Also, tumor xenografts grow more rapidly in *Sparc* null mice than in their wild-type littermates.^{15,16} These results point to a role for SPARC as a tumor suppressor in a variety of solid tumors.

Although its function in hematopoiesis has not been studied, the localization of the *SPARC* gene on chromosome 5q31, a region that is frequently deleted in AML and myelodysplastic syndromes, would be consistent with a tumor suppressor role for this protein in myeloid malignancy.¹⁷ *SPARC* is expressed by a variety of tissues including osteoblasts, adipocytes, megakaryocytes and Schwann cells and is a component of platelet α -granules that is released upon platelet activation.¹⁸ Aside from this, little is known about the regulation of *SPARC* expression in normal or malignant hematopoiesis. Here, we report that *SPARC* is expressed in normal CD34+ hematopoietic progenitors and in the leukemic blasts of most AML patients but is transcriptionally silenced in AML patients and cell lines with rearrangement of the *MLL* gene (*MLL*^R). Consistent with its effects on some human solid tumor cell lines, addition of exogenous purified SPARC to *MLL*^R AML cell lines specifically inhibited their proliferation. Although the mechanism for silencing of *SPARC* expression in *MLL*^R AML patients remains unclear, our results suggest that lack of SPARC protein could play a role in the pathogenesis of this disease.

Materials and methods

Analysis of microarray data

Detailed descriptions of patient characteristics, sample preparation, hybridization and data acquisition have been published

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previously¹⁹ and are available at the Stanford Microarray Database (<http://genome-www5.stanford.edu/>). The mean normalized log₂ Cy5:Cy3 ratios from the pediatric and adult datasets were analyzed as two class unpaired data using significance analysis of microarrays (SAM) with K-nearest neighbor imputation, 10 neighbors and 100 permutations.²⁰

Cells and culture conditions

Cryopreserved CD34 selected (80–97% pure) human cord blood (CB) cells were provided by the Translational Trials Development and Support Lab of the Cincinnati Children's Hospital Research Foundation under an IRB approved protocol. After thawing, cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 20% fetal or bovine calf serum, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) 1 × insulin, transferrin, selenium (ITS) (Mediatech, Inc. Herndon, Virginia, VT, USA), 0.1 mM β-mercaptoethanol, 20 ng/ml each of Flt3-Ligand, stem cell factor (SCF), thrombopoietin (TPO), interleukin 6 (IL-6) and 10 ng/ml IL-3 (Peprotech, Rocky Hill, NJ, USA). Preparation of RNA, protein or SPARC treatment were performed between days 3 and 7 of culture when CD34+ cells make up >50% of the culture (data not shown). AML cell lines were obtained from the American Type Culture Collection (<http://www.atcc.org>) or the German National Resource Center for Biological Material (<http://www.dsmz.de>). The relevant biological features of these cell lines have been described by Drexler and MacLeod²¹ (and references therein) and are summarized in Table 1. Cells were cultured in RPMI 1640 medium supplemented with 10% bovine calf serum, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 1 × ITS. For demethylation experiments, cells were cultured in the presence or absence of 1 μM 5-aza-2'-deoxycytidine (DAC) (SuperGen, Dublin, CA, USA) for 3 days. Fresh drug was added each day by adding 1 μl of a 10 mM DAC stock in phosphate buffered saline (PBS) per 1 ml of culture. SPARC treatment was performed by adding exogenous purified platelet SPARC (Haematologic Technologies Inc, Essex Junction, VT, USA) to cells in culture on day 1 of a 3-day incubation. Viability was assessed on day 3 by MTT assay.

Quantitative real-time RT-PCR

Total RNA was prepared from cells in the log phase of growth using the RNeasy Kit (Qiagen, Valencia, CA, USA) and reverse transcribed using the SuperScript First-Strand system (Invitrogen, Carlsbad, CA, USA) with random hexamer primers. Quantitative real-time reverse transcriptase mediated polymerase chain reaction (Q-RT-PCR) was performed using an ABI Prism 7700 Sequence Detection System. Triplicate reactions (25 μl) were set up using 2.5 × SYBR Green Master Mix (Applied Biosystems,

Foster City, CA, USA) according to the manufacturer's recommendations. Relative quantitation of SPARC was carried out as described in User Bulletin #2 ABI Prism 7700 Sequence Detection System using c-ABL as the endogenous control. Total RNA from the K562 cell line was used to establish a standard curve and the Kasumi-1 cell line was used as the calibrator sample. SPARC primer sequences were 5'-ATC TTC CCT GTA CAC TGG CAG TTC-3' (forward) and 5'-CTC GGT GTG GGA GAG GTA CC-3' (reverse). c-ABL primer sequences were 5'-TGC CCA GAG AAG GTC TAT GAA CT-3' (forward) and 5'-AAC ATT GTT TCA AAG GCT TGG TG-3' (reverse). For Q-RT-PCR analysis of SPARC RNA in patient specimens, GAPDH was used as the endogenous control as described in Lacayo et al.¹⁹

Western blot analysis

Whole-cell extracts were prepared in SDS lysis buffer. Alternatively, cell culture supernatants were concentrated by using Centricon (Millipore, Bedford, MA, USA) filters as instructed by the manufacturer. Briefly, the filtrate from a Centricon-100 spin column was loaded onto a Centricon-10 column and the retentate was collected when the volume was reduced by approximately 100-fold. Equivalent amounts of whole-cell lysate or concentrated culture supernatants (as measured by Bradford assay) from each cell line underwent electrophoresis in a 10% SDS polyacrylamide gel. The proteins were transferred to nylon membranes and incubated with antibody to SPARC (Haematologic Technologies, Essex Junction, VT, USA) at a 1:5000 dilution. Duplicate membranes were incubated with the pan-actin C4 antibody (Seven Hills Bioreagents, Cincinnati, OH, USA). Membranes were then incubated with secondary anti-mouse IgG1 conjugated to HRP and detected with the Super-Signal West Femto Kit (Pierce, Rockford, IL, USA).

Bisulfite genomic sequencing

Genomic DNA was prepared using the QIAamp DNA Blood Mini-kit (Qiagen). Approximately 1 μg of DNA was bisulfite converted using the EZ Kit (Zymo Research Corp., Orange, CA, USA). Converted DNA (top strand) was PCR amplified using published primer sequences and conditions.¹⁰ PCR products were cloned using a Topo TA cloning kit (Invitrogen) and individual clones were sequenced by the Cincinnati Children's Hospital Genomics Core, which used an ABI 3700 DNA analyzer.

Proliferation assays

MTT assays were performed on cells cultured in 100 μl of media, with or without drug, in 96-well plates. At the end of this incubation period, 10 μl thiazolyl blue tetrazolium (5 mg/ml in PBS) (Sigma, St Louis, MO, USA) was added to each well and cells were incubated at 37°C for 3–4 h. At the end of this incubation, MTT formazan was solubilized in 100 μl 0.1% SDS and 1 N HCl overnight. The absorbance at 562 nm (minus the background absorbance at 650 nm) was measured using an automated plate reader.

Cell cycle analysis

SPARC-treated or -untreated cells were incubated with 10 μM bromodeoxy-uridine (BrdU) for 30 min at 37°C then fixed with cold 70% ethanol and permeabilized with 2 N HCl and 0.5% Triton X-100. Cells were then stained with anti-BrdU antibody conjugated to fluorescein isothiocyanate, washed and resus-

Table 1 AML cell lines used in this study

Cell line	Translocation	Fusion expressed	MLL genotype
Kasumi-1	t(8;21)	AML1-ETO	wt
ME-1	Inv(16)	CBFβ-MYH11	wt
KG1a	NA	None	wt
ML-2	t(6;11)	MLL-AF6	R
MV411	t(4;11)	MLL-AF4	R
THP-1	t(9;11)	MLL-AF9	R

Relevant cytogenetic features, associated fusion gene/protein and MLL wild-type (wt) or rearranged (R) are listed for each cell line used in this study.

pendent in 7-amino-actinomycin D. BrdU incorporation and DNA content were detected by flow cytometry using a FACSCANTO cytometer (Beckton-Dickinson, Mountain View, CA, USA).

Results

SPARC is underexpressed in AML patients and cell lines with *MLL* gene rearrangements

Chromosomal translocations at 11q23 disrupt the gene encoding *MLL*, a positive epigenetic regulator of transcription.^{22–24} A possible mechanism whereby these rearrangements could contribute to the pathogenesis of AML is via the inappropriate silencing of genes subordinate to *MLL*, some of which may control proliferation and survival. To identify genes that were underexpressed specifically in patients with *MLL* gene rearrangements, we analyzed gene expression profiles at diagnosis in bone marrow specimens from 70 pediatric AML patients treated on the Pediatric Oncology Group (POG) study 9421. These samples included 13 patients with *MLL* gene rearrangements. Four of these patients had a t(9;11), three had a t(11;19) and one each had a t(1;11), t(11;17) and t(10;11). Three patients had disruption of the *MLL* gene detectable by fluorescence *in situ* hybridization. The non-*MLL* rearranged (*MLL*^{wt}) patients included 45 with normal or complex karyotypes (N/C), two with t(8;21) and 10 with inv(16). Gene expression profiling was performed on high-density cDNA microarrays representing 23 867 genes as described by Lacayo et al.¹⁹

We used SAM to identify genes whose expression differed by more than twofold between the *MLL*^R and the *MLL*^{wt} groups.²⁰ Forty genes were found to be differentially expressed between these groups, with a median false discovery rate of 0.75% (data not shown). 31 genes, including *SPARC*, were expressed at

levels more than twofold lower in the *MLL*^R patients as compared with the *MLL*^{wt} patients. Although there was a lower mean level of *SPARC* expression in the *MLL*^R patients as compared with the N/C group, the most significant difference ($P=0.001$) was between the *MLL*^R and inv(16) patients (Figure 1a). Two patients with t(8;21) also expressed *SPARC* at levels comparable to the inv(16) patients. Analysis of independent AML microarray data sets from the literature^{25,26} also revealed mean expression levels for *SPARC* that were 2- to 4-fold lower in *MLL*^R patients as compared with other cytogenetically defined subsets such as inv(16) and t(8;21) (Figure 1b and c). The consistency of this observation is particularly striking considering that one of these studies used the same cDNA microarray platform as we used but analyzed adult AML patients²⁵ whereas the other study examined pediatric AML patients but used an oligonucleotide microarray platform.²⁶ To determine if the relatively low level of *SPARC* expression in *MLL*^R cells reflected physiologic downregulation associated with lineage commitment, we analyzed *SPARC* expression in relation to French American British (FAB) subtype in the POG 9421 patients. We found no statistically significant association between *SPARC* expression and FAB subtype when *MLL*^R and inv(16) patients were excluded (data not shown). Low *SPARC* expression, as detected by microarray analysis, thus appears to be specifically associated with the presence of an *MLL* gene rearrangement.

To confirm the microarray results, we performed Q-RT-PCR on RNA from selected POG 9421 patient samples and from CD34+ CB cells using primers for *SPARC*. As a parallel control, we also performed Q-RT-PCR using primers for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which did not vary significantly between patients on the microarrays. These studies showed that *SPARC* expression, normalized to *GAPDH*, in patients with inv(16) and normal or complex karyotypes was

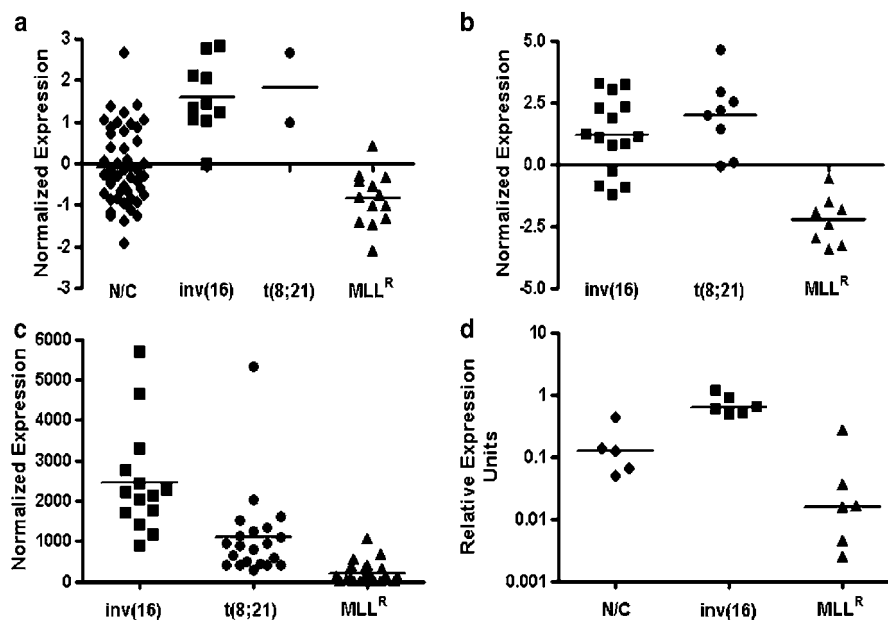


Figure 1 *SPARC* is underexpressed in AML patients with *MLL* rearrangements. Mean (0) normalized \log_2 Cy5: Cy3 ratios for *SPARC* (IMAGE # 487 878) in diagnostic bone marrow samples from pediatric (POG 9421) AML patients¹⁹ (a) or adult AML patients²⁵ (b) hybridized to cDNA microarrays produced by the Stanford Functional Genomics Core. (c) Scaled (500) signal intensity from the *SPARC* (Affymetrix 200665_s_at) probeset in samples from pediatric AML patients.²⁶ Each point represents one patient. (d) Quantitative real-time RT-PCR analysis of *SPARC* expression (normalized to *GAPDH*) in patients from the POG 9421 study. Five patients had normal or complex karyotypes (N/C), six patients had inv(16) and six patients had *MLL* rearrangements. Unit less expression values are relative to the mean *SPARC* expression (normalized to *GAPDH*) of two separate CD34+ cord blood (CB) samples (arbitrarily set to 1).

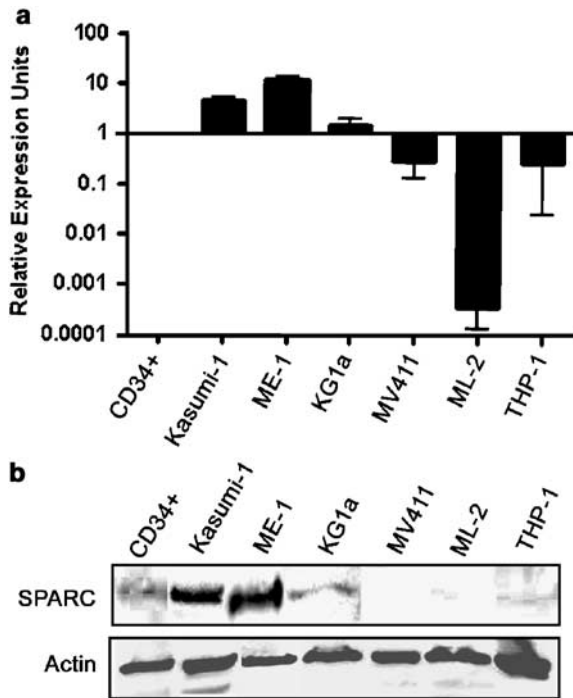


Figure 2 SPARC is silenced in AML cell lines. (a) Quantitative real-time RT-PCR analysis of *SPARC* expression in CD34+ CB and AML cell lines. Bars represent the mean and standard deviation of at least three independent experiments. Expression in CD34+ CB was set to one in each experiment; values for cell lines are relative to CD34+ CB. (b) Western blot analysis of whole-cell extracts with anti-SPARC antibody (upper panel) or with pan-actin antibody, which served as a loading control.

roughly equivalent to that of normal CD34+ progenitors while mean expression in *MLL*^R patients was reduced by as much as two orders of magnitude (Figure 1d). We next examined *SPARC* expression at the RNA and protein level in a number of *MLL*^R and *MLL*^{wt} AML cell lines. Relative to normal CD34+ CB cells, AML cell lines with t(4;11) (MV411) or t(9;11) (THP-1) expressed 3- to 4-fold lower levels of *SPARC* RNA (Figure 2a). *SPARC* transcripts were 500- to 1000-fold lower by Q-RT-PCR in the t(6;11) bearing cell line ML-2 than in CD34+ CB. In contrast, the t(8;21) bearing AML cell line Kasumi-1 expressed 4- to 5-fold more and the inv(16) bearing cell line ME-1 almost 10-fold more *SPARC* RNA than CD34+ CB. KG1a cells, which have no known recurring translocation, expressed levels of *SPARC* RNA that were comparable to those observed in CD34+ CB. By Western blot, no *SPARC* protein was detected in extracts of MV411, ML-2 or THP-1 whereas the protein was easily detectable in Kasumi-1 and ME-1 extracts and detectable at lower levels in CD34+ and KG1a cells (Figure 2b). These results indicate that *SPARC* RNA and protein are expressed at low to undetectable levels in AML cells with *MLL* gene rearrangements.

SPARC inhibits the growth of AML cell lines with *MLL* gene fusions

In addition to its growth-inhibitory effects on normal endothelial cells, *SPARC* has been shown to induce growth arrest and apoptosis in a number of different tumor cell lines.^{10,11,14} We hypothesized that *MLL*^R AML cells might also be sensitive to this growth-inhibitory effect, whereas cells that express *SPARC*

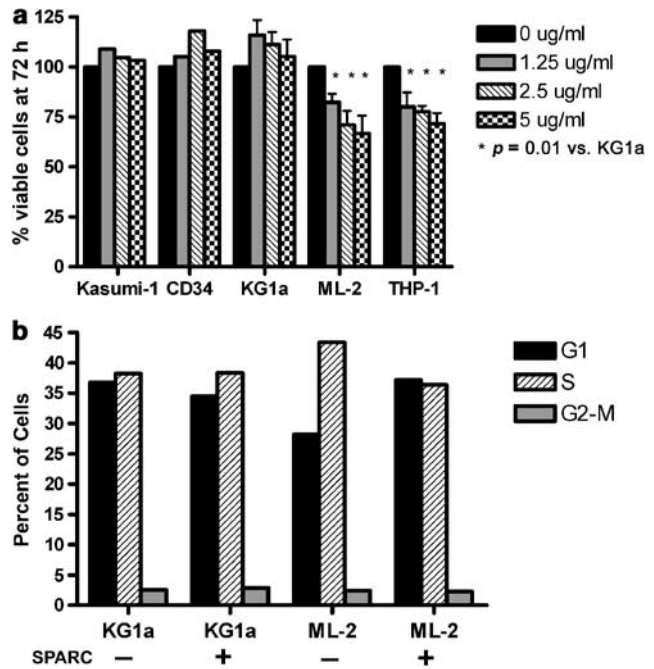


Figure 3 Growth and cell cycle progression are inhibited by SPARC. (a) Effect of exogenous SPARC on survival of AML cell lines and normal CD34+ CB cells measured by an MTT assay. Each bar represents the mean and standard deviation of three separate experiments for ML-2, THP-1 and KG1a. Kasumi-1 and CD34+ CB cells were tested twice. Representative results are shown. (b) Cell cycle distribution of SPARC-treated and -untreated cells as measured by flow cytometry after BrdU incorporation and 7-AAD staining.

would be resistant. To test this hypothesis, we added exogenous purified *SPARC* protein to cells in culture at a range of concentrations shown to inhibit the proliferation of vascular endothelial cells.²⁷ *SPARC* inhibited the growth of ML-2 and THP-1 cells by 30–40% in a concentration-dependent manner, after a 72-h incubation period (Figure 3a). The growth of Kasumi-1, KG1a and normal CD34+ CB cells was not affected by *SPARC* at these concentrations. Annexin staining of *SPARC*-treated ML-2 and THP-1 cells did not reveal apoptosis above background levels (data not shown). *SPARC* has been shown to inhibit cell cycle progression in vascular endothelial cells.²⁷ To determine if the growth-inhibitory effect of *SPARC* in *MLL*^R AML cells was due to effects on the cell cycle, we labeled *SPARC*-treated and -untreated KG1a and ML-2 cells with BrdU and 7-amino-actinomycin D. Flow cytometry revealed a 7–8% reduction in the percentage of cells in Sphase with a concomitant increase in the percentage of cells in G1 in the *SPARC* sensitive ML-2 cells but no change in the *SPARC* resistant cell line KG1a was observed (Figure 3b). These results suggest that *SPARC* inhibits progression from G1 to Sphase in the ML-2 cells. This inhibition could account for the reduced growth rate of these cells when exposed to exogenous *SPARC*.

SPARC silencing is associated with promoter methylation in *MLL*^R cell lines but not in patient cells

The *SPARC* gene is a target of epigenetic gene silencing by promoter methylation in pancreatic carcinoma cells.¹⁰ To determine whether DNA methylation was the basis for the loss of *SPARC* expression in AML cells with *MLL* rearrangements, we

performed bisulfite genomic sequencing of the *SPARC* promoter, beginning with the AML cell lines. We focused our analysis on 12 CpG dinucleotides spanning a 404-bp region that includes the transcriptional start site, the first exon and part of the first intron. Methylation of these 12 CpG dinucleotides was shown to correlate with *SPARC* silencing in pancreatic carcinoma cells.¹⁰ Sequencing of multiple clones revealed a nearly complete lack of cytosine methylation surrounding exon 1 in the *SPARC*-expressing cell line KG1a and extensive methylation in the nonexpressing cell line ML-2 (Figure 4a). A similar association between *SPARC* expression and promoter methylation was also observed for Kasumi-1 and THP-1 cells (data not shown). Treatment of cells with the DNA demethylating agent DAC resulted in only one-third of the clones becoming unmethylated (data not shown). Despite this modest decrease in methylation, DAC treatment resulted in a dramatic increase in expression of *SPARC* in ML-2 cells that was detected by Q-RT-PCR and Western blot (Figure 4b and c). The slight decrease in

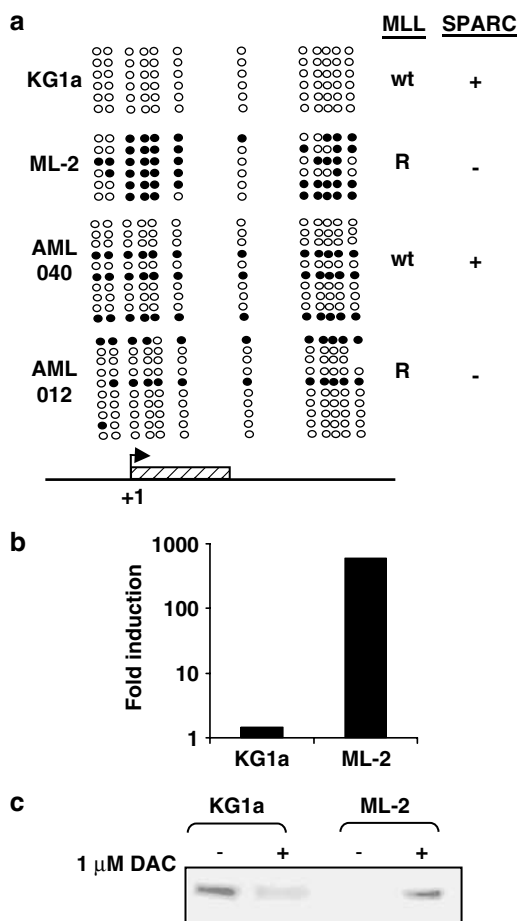


Figure 4 *SPARC* silencing is associated with promoter methylation in AML cell lines but not in patient cells. (a) Results of bisulfite sequencing of *SPARC* 5' flanking sequences, transcriptional start site (+1) first exon (Ex. 1) and 5' end of the first intron. Circles represent CpG dinucleotides revealed by bisulfite conversion to be methylated (filled) or unmethylated (open). Each row depicts an individual cloned bisulfite modified PCR product from AML cell lines (KG1a, ML-2) or from representative patient samples (AML 040, 012). (b) Increase in *SPARC* RNA measured by qRT-PCR in DAC-treated and -untreated cells. Bars represent the mean and standard deviation of at least three independent experiments. (DAC) for 72 h in culture. (c) Western blot analysis of *SPARC* protein in concentrated cell culture supernatants of DAC-treated and -untreated cells.

SPARC protein expression by DAC-treated KG1a cells was also observed at the RNA level in some but not all Q-RT-PCR experiments. This could reflect terminal differentiation or loss of viability after DAC treatment in the KG1a cells. These results suggest that the loss of *SPARC* expression in ML-2 and THP-1 cells is the result of transcriptional repression associated with promoter methylation. In contrast to the cell lines, bisulfite sequencing of more than 50 independent clones representing six *MLL*^R patients from the POG 9421 study did not reveal increased methylation at the *SPARC* promoter as compared to patients with *inv(16)* despite significant differences in *SPARC* expression (Figure 4a). The lack of *SPARC* expression in these *MLL*^R patients thus cannot be explained on the basis of promoter methylation.

Discussion

We have shown that *SPARC*, a matricellular glycoprotein and putative tumor suppressor, is underexpressed at the transcriptional level in *MLL*^R AML patients. This subset of patients experiences poor clinical outcomes largely as a result of resistance to chemotherapy.¹ Recently, loss of *SPARC* expression has been identified as a contributing factor to acquired chemotherapy resistance in colon carcinoma cells.²⁸ In the POG 9421 dataset, low *SPARC* expression is not, by itself, associated with decreased event free or overall survival. Nevertheless, it is intriguing to speculate that the lack of *SPARC* protein may contribute to the clinical behavior of *MLL*^R AML.

Underexpression of *SPARC* in the cells of *MLL*^R AML patients, as compared to AML with normal or complex karyotypes, *inv(16)* or *t(8;21)* was a consistent observation across independent adult and pediatric microarray datasets using different microarray platforms. This difference in *SPARC* expression was most pronounced when comparing *MLL*^R patients to patients with *inv(16)* or *t(8;21)*. However, *SPARC* expression in *MLL*^R AML was also low in comparison to normal CD34+ hematopoietic progenitors as measured by Q-RT-PCR. This argues that the differences in *SPARC* expression revealed by microarray analysis of patient samples reflects a lack of expression in *MLL*^R patients rather than mere overexpression in the *inv(16)* group. From these data, we conclude that underexpression of *SPARC* is a robust transcriptional feature of AML with *MLL* gene rearrangements. It remains unknown whether rearrangement of the *MLL* gene is causally related to the decreased expression of *SPARC*. Although HOX genes are the best-known targets of *MLL*, it is likely that there are many non-HOX genes that are subject to epigenetic control by this protein. As *MLL* gene fusions lead to loss of normal *MLL* function, as well as gain of transforming activity, these rearrangements could result in inappropriate silencing of some *MLL* targets. Alternatively, *SPARC* silencing could be a downstream effect of an *MLL* target gene.

Our studies showed that the loss of *SPARC* expression in AML cell lines with *MLL* rearrangements is associated with promoter methylation. Treatment of AML cell lines with DAC, a DNA demethylating agent, led to a dramatic increase in the expression of *SPARC* RNA and protein. The relatively modest degree of demethylation observed after DAC treatment in relation to the large magnitude of the transcriptional response (Figure 4b and c), has been described previously for the p16/CDKN2 gene, which is heavily methylated in a number of cancer cell lines.^{29,30} We did not, however, find dense methylation of the *SPARC* promoter in bone marrow samples

obtained from MLL^R pediatric AML patients. It is possible, although unlikely, that this absence of dense methylation reflects the difference between the homogeneity of the cell lines and the relative heterogeneity of the bone marrow specimens. The average blast percentage in the diagnostic bone marrow specimens from the POG 9421 study was 86%; therefore, contaminating normal bone marrow cells were not sufficient to obscure a major difference in methylation. A more likely explanation is that CpG methylation was acquired by the cell lines in culture and that the SPARC promoter is silenced in primary cells by other means. For example, silencing of SPARC in the primary AML blasts could be mediated by hypoacetylation and/or methylation of specific residues in histones. These modifications often precede CpG methylation, which is a final and irreversible epigenetic event.^{31–34} The limited availability of MLL^R patient specimens precluded our ability to address this possibility directly by chromatin immunoprecipitation. We have, however, observed that SPARC expression is downregulated without DNA methylation in long-term cultures of CD34+ CB cells retrovirally transduced with MLL-AF9 (J DiMartino, unpublished observation). Future studies will use this model system to address the mechanism for transcriptional changes associated with MLL fusion protein expression.

Our data are consistent with a model in which the loss of SPARC expression provides a growth advantage to MLL^R myeloid leukemia cells through the loss of autoregulatory signals that inhibit proliferation. The ability of SPARC to upregulate the expression of TGF- β ^{35,36} suggests that SPARC could play a role in maintaining HSC and possibly leukemic cells in a quiescent state. Furthermore, by blocking signals from the vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF)^{37–39} SPARC may be depriving leukemic cells of survival signals. Loss of SPARC could, therefore, enable MLL^R AML cells to avoid apoptosis in response to chemotherapy. In addition to its potential cell-autonomous effects on leukemic blasts, SPARC expression may limit the progression of leukemia by inhibiting neoangiogenesis, which plays an important role in the pathogenesis of leukemia.⁴⁰

It is conceivable that the *in vitro* antiproliferative effect of exogenous SPARC that we have described could eventually be exploited therapeutically by administering recombinant SPARC or one of its derivative peptides to AML patients. The feasibility of this approach is supported by experiments demonstrating that subcutaneously administered SPARC can inhibit the growth of neuroblastoma xenografts in mice.¹⁴ More recent data, showing that exogenous SPARC can reverse acquired resistance to chemotherapeutic agents²⁸ further suggests that SPARC could increase the effectiveness of anthracyclines and other agents that are active in AML. We are currently investigating this hypothesis.

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