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# A vicious loop of fatty acid-binding protein 4 and DNA methyltransferase 1 promotes acute myeloid leukemia and acts as a therapeutic target

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## Abstract

Aberrant DNA methylation mediated by deregulation of DNA methyltransferases (DNMT) is a key hallmark of acute myeloid leukemia (AML), yet efforts to target DNMT deregulation for drug development have lagged. We previously demonstrated that upregulation of fatty acid-binding protein 4 (FABP4) promotes AML aggressiveness through enhanced DNMT1-dependent DNA methylation. Here we demonstrate that FABP4 upregulation in AML cells occurs through vascular endothelial growth factor (VEGF) signaling, thus elucidating a crucial FABP4-DNMT1 regulatory feedback loop in AML biology. We show that FABP4 dysfunction by its selective inhibitor BMS309403 leads to downregulation of DNMT1, decrease of global DNA methylation and re-expression of $p15^{INK4B}$ tumor suppressor gene by promoter DNA hypomethylation *in vitro, ex vivo* and *in vivo*. Functionally, BMS309403 suppresses cell colony formation, induces cell differentiation, and, importantly, impairs leukemic disease progression in mouse models of leukemia. Our findings highlight AML-promoting properties of the FABP4-DNMT1 vicious loop, and identify an attractive class of therapeutic agents with a high potential for clinical use in AML patients. The results will also assist in establishing the FABP4-DNMT1 loop as a target for therapeutic discovery to enhance the index of current epigenetic therapies.



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**Author contributions**

S.J.L. and B.L. designed research; F.Y., N.S., J.X.P., N.Z., and Y.W.Z. performed research; A.A. and M.R.L. provided the leukemia patient samples; S.J.L., B.L., M.R.L., F.Y., A.M. B., and A.A. analyzed data and wrote the paper; S.J.L. conceived ideas and oversaw the entire research project.

## Introduction

Acute myeloid leukemia (AML) is a highly aggressive hematologic malignancy characterized by the swift uncontrolled growth of immature myeloblasts. It is a lethal disease that lacks effective treatment. Although the precise molecular causes that are responsible for AML development and disease progression are unclear, it seems to result from an interplay of genetic and environmental factors that are largely unidentified. The fatty acid-binding protein (FABP) 4, also known as adipocyte FABP (A-FABP) or aP2, is a master regulator of lipid metabolism and significantly implicated in inflammation and cancers.[1, 2] FABP4 is postulated to be predominantly expressed in adipocytes and macrophages and to act as an adipokine.[3] FABP4 production can be modulated by environmental factors, including dietary intake,[4] which is consistent with the observations that elevation of circulating FABP4 levels is associated with diet-induced obesity.[5, 6] FABP4 is also reported to be abundantly expressed in several types of cancer cells.[6–9] In agreement with the above expression patterning, FABP4 upregulation in obese host and in leukemia cells enhances AML disease progression in both a cell-autonomous and cell-non-autonomous manner.[6] Thus, the specific mechanisms promoting FABP4 expression merit systematic exploration.

Cancer is well known to be a genetic disease. However, mounting evidence suggests that altered epigenetic phenotypes (e.g., aberrant DNA methylation) occur more frequently than genetic mutations, and could serve as an alternative etiological mechanism to a genetic mutation for the identical disease.[10] DNA methylation involves the addition of a methyl group to the number 5-carbon of the cytosine pyrimidine ring, which is achieved by a cooperative effect among DNA methyltransferases (DNMTs) 1, 3a and 3b.[11, 12] DNMT overexpression is frequent in leukemia cells and aberrant DNA methylation serves as a key hallmark of leukemogenesis.[6, 12–14] But efforts to target DNMT deregulation for drug development have lagged. Abnormal DNA methylation is mainly attributed to cell-autonomous signaling, such as microRNAs,[11, 14] nucleolin,[12] Sp1/NFkB nuclear factor kappa B),[15] AML1/ETO,[13] ten-eleven translocation (TET) family,[16] or cytidine deaminases.[17] In support of this idea, our recent discoveries identified cellular FABP4 as an additional cell-autonomous member of the DNA methylation regulator family. More importantly, we demonstrated host/environmental FABP4 as a hitherto unknown cell non-autonomous factor governing the DNA methylation machinery, which acts partially through the IL-6/STAT3 pathway.[6] We propose that FABP4 is a novel epigenetic member that can be pharmacologically targeted for AML intervention. Here, using comprehensive molecular, cellular and animal studies, we demonstrate that DNMT1 feeds back to upregulate FABP4 through hyperactive VEGF signaling. We show that BMS309403, a selective FABP4 inhibitor, can induce AML cell differentiation and block AML progression in leukemia-bearing mice. Mechanistic investigations disclose that BMS309403-mediated growth arrest of AML cells takes place through the reduction of DNMT1-dependent DNA methylation. These data suggest that targeting the FABP4-DNMT1 loop is a novel strategy for developing next-generation DNA hypomethylating agents to improve the dismal outcomes of conventional epigenetic compounds.

## Materials and Methods

### Cell lines, plasmids, reagents and patient samples

The 293T, MV4-11, Kasumi-1, THP-1 and C1498 cell lines were obtained from American Type Culture Collection. The SKNO-1 cell line was kindly provided by Dr. Clara Nervi (University of Rome, Rome, Italy). Cell lines were newly purchased with no further testing for mycoplasma. The primary blasts from AML patients were obtained from the Tumor Tissue/Biospecimen Bank of Mayo Clinic. All patients signed an informed consent document approved by the Mayo Clinic Institutional Review Board before entering the study. Details are in Supplementary Materials and Methods.

### Western blotting

The whole cellular lysates were prepared by harvesting the cells in 1 × cell lysis buffer and Western blotting was performed as previously described.[6, 11–13, 18] The following antibodies were purchased from Abcam (Cambridge, MA): anti-IL-6 (ab6672, 1:500), and anti-VEGF (ab46154, 1:500). Anti-β-actin (sc-1616, 1:1000) and anti-mouse-DNMT1 (sc-52919, 1:1000) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-STAT3 (9139, 1:1000) and anti-phospho-STAT3 (Tyr705, 9131, 1:1000) were from Cell Signaling Technology (Danvers, Massachusetts). Anti-human-FABP4 (AF3150, 1:1000) and anti-mouse-FABP4 (AF1443, 1:500) were from R&D systems (Minneapolis, MN) and the anti-human-DNMT1 (M0231L, 1:500) was from New England Biolabs (Ipswich, MA).

### DNA Dotblotting

Genomic DNA was purified using DNA Blood/Tissue Kit (QIAGEN, Germantown, MD), denatured and subjected to Dotblotting analysis using a 5mC antibody (39649, 1:2500, Active Motif, Carlsbad, CA) as previously described.[6, 11–13, 18] The DNA spotted membrane was stained with 0.02% methylene blue (Sigma) in 0.5 M sodium acetate (pH 5.0) for DNA loading control.

### Bisulfite sequencing

The bisulfite sequencing was performed as previously described.[6, 11, 13, 18, 19] Briefly, 2 μg DNA was converted and purified using the EpiTect Bisulfite Kit (59824, QIAQEN). The region (−4 to +247) within the *p15*$^{INK4B}$ promoter was PCR-amplified from bisulfite-treated DNA. The primers are listed in Supplemental Table. The PCR products were subcloned using the TA Cloning® Kit (450046, Invitrogen) and sequenced by Genewiz (South Plainfield, NJ).

### RNA isolation, cDNA preparation and quantitative PCR (qPCR)

RNA was isolated using the miRNeasy Kit (QIAGEN) and reverse transcription for cDNA was performed using the SuperScript® III First-Strand Synthesis System (18080-051, Invitrogen). TaqMan qPCR (4331182, Applied Biosystems) was performed to measure *DNMT1* expression, and SYBR-Green qPCR (4309155, Applied Biosystems, Foster City, CA) was used for other genes. The primers are listed in Supplemental Table. The *18S* levels were used for normalization and target expression was analyzed using the ΔCT approach.

### Cytospin/Wright–Giemsa staining, cell differentiation, wound-healing, and colony-forming assays

All these assays were performed as previously described.[6, 11–13, 18, 19] Details are in Supplementary Materials and Methods.

### Animal studies

All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Minnesota and were in accordance with the US National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. Details are in Supplementary Materials and Methods.

### Statistical analysis

All the graphs were generated using Student's t-test, but the Kaplan–Meier survival curves were created by log-rank test. Correlation data were acquired using the Pearson's correlation coefficients. Details are in Supplementary Materials and Methods.

## Results

### FABP4 and SFAs cooperatively augment leukemia cell colony formation

While the serum levels of both saturated fatty acids (SFAs) and FABP4 are high in obesity compared to lean subjects,[5, 6] an experimental demonstration of a SFA-FABP4 functional association remains undocumented. To this end, bone-marrow derived cells were exposed to stearic acid (SA) or palmitic acid (PA), which are the common SFAs in high-fat diets. FABP4 expression was significantly increased at both the mRNA (Figure 1a) and protein levels (Figure 1b) in the presence compared to the absence of SA and PA, which support the existence of a causal relationship between SFAs and FABP4. Given the worse prognosis of leukemia in obese people and mice,[6] we speculated that the interactions between excess SFAs and higher levels of FABP4 account for a more aggressive form of leukemia. As a proof of concept, we first measured the growth of FABP4 wild-type (*FABP4*$^{+/+}$) and knockdown (*FABP4*$^{-/-}$) macrophages in the presence or absence of SFAs. We found that *FABP4*$^{+/+}$ migrated much faster than *FABP4*$^{-/-}$ macrophages in the presence of SA, indicating that genetic deficiency of *FABP4* curbs the response of the cells to SFA stimulation (Figure 1c). This result suggests that FABP4 is required for SA-augmented cell proliferation, which is aligned with the role of FABP4 as a lipid transporter.[1, 2] We then treated MV4-11 or C1498 AML cells with SA alone or with the FABP4 protein, and found that the cells treated with the combination displayed a more robust colony formation than observed with either agent alone (Figure 1 d and e). Confocal microscopy analysis revealed that a combination of SA and FABP4 greatly enhanced lipid accumulation in leukemia cells (Figure 1f). Notably, cellular apoptosis was observed and proliferation was impaired when SA concentrations were above 75 μM (not shown), consistent with previous reports,[20, 21] which showed that higher lipid doses could invoke cytotoxicity. Finally, to understand the mechanisms by which SFAs contribute to leukemia growth, we treated MV4-11 cells with suboptimal dose of either stearic acid (SA) alone or plus the FABP4 protein, given that FABP4 is a crucial lipid transporter. We found that, in the presence of the FABP4 protein,

SA treatment increased the protein expression and phosphorylation of STAT3, a *DNMT1* transactivator, with a concurrent DNMT1 upregulation (Figure S1 a and b). In line with this, the combination of SA with FABP4 protein induced higher levels of DNA methylation compared to single agent groups (Figure S1c), collectively supporting the notion that DNMT1 upregulation by stearic acid could be an important mechanism underlying SFA-promoted leukemia growth. Taken together with the results of our previous studies,[6] these data further support a role of FABP4 in AML disease progression.

## DNMT1 induces ectopic expression of *FABP4* in leukemia cells

To understand the molecular mechanisms underlying *FABP4* upregulation, we focused on DNMT1, because *FABP4* expression parallels *DNMT1* expression in leukemia cells and in obesity.[6] In accordance, shRNA-triggered *DNMT1* knockdown in C1498, MV4-11 and Kasumi-1 cells resulted in an apparent decrease in FABP4 expression (Figure 2 a and b). Consistently, exposure of leukemia cells to decitabine, a classical DNMT1 inhibitor,[22] led to substantial downregulation of FABP4, but not FABP5 (Figure 2c). To further validate DNMT1 in *FABP4* regulation, we transfected 293T or Kasumi-1 cells with *DNMT1* expression vectors, and initially verified the promoting properties of *DNMT1* overexpression (Figure S2a) in DNA methylation (Figure S2*b*) and cell migration (Figure S2 c and d), supporting our previous studies showing that *DNMT1* knockdown impairs clonogenic potential of AML cells.[6] As expected, enforced *DNMT1* expression led to a concurrent increase in *FABP4* levels (Figure 2d; Figure S2e). Because a cooperation among DNMT1, 3a and 3b determines DNA methylation status,[11, 12] we further analyzed GEO datasets containing 242 AML patients,[23] but did not find a parallel association between FABP4 with DNMT3a or DNMT3b (Figure 2 e and f), providing further support of DNMT1 in governing FABP4 gene expression. Considering a positive role of FABP4 in regulating the *DNMT1* gene,[6] these results suggest that DNMT1 and FABP4 are engaged in a reciprocal regulatory loop in AML cells.

## VEGF mediates DNMT1-upregulated *FABP4* expression

Because limited numbers of CpG within 3kb up- or down-stream of the transcription start site of human or mouse *FABP4* promoter were identified (not shown), we reasoned that DNMT1 might not directly regulate *FABP4* by promoter methylation. We targeted vascular endothelial growth factor (VEGF), because a) VEGF is an important obesity-leukemia associated molecule;[24–28] b) compared to lean subjects, VEGF is expressed at a higher level in serum (Figure S3a) and BM (Figure S3b) from leukemia-bearing obese mice, in line with more aggressive leukemia growth in obese patients and mice;[6] c) the analysis of GSE12417[29] showed that AML patients with higher *VEGF* levels displayed shorter survival times compared with those with lower *VEGF* (Figure 3 a and b). These *in vivo* observations were experimentally verified by showing that treatment with VEGF recombinant protein largely increased colony number of C1498, MV4-11 and Kasumi-1 cells (Figure 3c). To this end, we manipulated *DMNT1* expression in AML or 293T cells, and observed that VEGF levels were decreased by *DNMT1* knockdown (Figure 3 d and e), but increased by *DNMT1* overexpression (Figure S3 c and d). Consistently, exposure of MV4-11 and Kasumi-1 cells to decitabine inhibited VEGF expression (Figure 3f). Collectively, these data support a positive regulatory role of DNMT1 in *VEGF* expression.

To validate the role of VEGF in FABP4 regulation, C1498, MV4-11 and Kasumi-1 AML cells were incubated with the VEGF protein. We found that FABP4 expression was increased in a dose-dependent manner (Figure 3g, Figure S4a), consistent with a previous report showing that FABP4 is induced by VEGFA in endothelial cells.[30] Further, treatment with the VEGF protein rescued *FABP4* downregulation driven by *DNMT1* depletion (Figure S4b), but attenuation of both *DNMT1* and *VEGF* induced a more pronounced suppression of *FABP4* production (Figure S4c). Finally, by analysis of GSE12417, we identified a significant positive correlation between *VEGF* and *FABP4* expression in AML patients (n = 242) (Figure S5). Thus, our data suggest that VEGF mediates, at least partially, the DNMT1-FABP4 feedback loop in AML cells.

## Pharmacological inactivation of FABP4 reduces DNMT1-dependent DNA methylation and restores the epigenetically-silenced *p15$^{INK4B}$* expression

Considering our current and previous findings[6] demonstrating the existence of an FABP4-DNMT1 loop in AML disease progression, pharmacological targeting FABP4 might be a valid approach to treat AML. BMS309403 has been reported to be a biphenyl azole inhibitor that specifically binds and inactivates FABP4.[31] Consistently, BMS309403 treatment specifically suppressed *FABP4*$^{+/+}$ macrophage migration while leaving the *FABP4*$^{-/-}$ macrophages largely unaffected (Figure S6 a and b), indicating that BMS309403 inhibitory effects were FABP4-dependent. Further, in support of the role of FABP4 in controlling the IL-6-STAT3-DNMT1 cascade,[6] exposure of AML cell lines to BMS309403 resulted in IL-6 reduction, STAT3 dephosphorylation and DNMT1 downregulation (Figure 4 a and b), followed by global DNA demethylation (Figure 4c). Notably, BMS309403-induced DNMT1 downregulation (Figure S7 a and b) and DNA demethylation (Figure S7 c and d) were rescued by the addition of the FABP4 protein into the culture medium, substantiating the idea that BMS309403-induced DNA hypomethylation is not off-target, but specific to FABP4 function.

TSGs (e.g., *p15$^{INK4B}$*) are frequently silenced epigenetically, and TSG-silencing predicts worse survival in leukemia.[6, 13] We used *p15$^{INK4B}$* as readout to determine whether BMS309403 changes promoter DNA methylation, because a literature review suggests that hypermethylation of the *p15$^{INK4B}$* gene is very common (> 50%), and is independently correlated with poor prognosis in patients with AML.[32–35] Our results indicated that *p15$^{INK4B}$* expression was upregulated in Kasumi-1, MV4-11 and C1498 cells in the presence compared to the absence of BMS309403 (Figure 4d). The bisulfite sequencing[11–13] disclosed that *p15$^{INK4B}$* promoter methylation was decreased by BMS309403 in MV4-11 (26% versus 2%) and Kasumi-1 (100% versus 91%) cells (Figure 4e), in agreement with the effects of siRNA-mediated *FABP4* downregulation.[6] To determine the therapeutic relevance of BMS309403 in human AML, we treated human patient (n = 8) or mouse (n = 3) primary cells with BMS309403 for 48 hours. Consistent with its ability to strongly disrupt the FABP4-DNMT1 loop in cell lines, BMS309403 markedly suppressed *DNMT1* expression (Figure 4f), lowered global DNA methylation (Figure S8a) and restored *p15$^{INK4B}$* expression (Figure S8b) in these primary cells. Bisulfite sequencing revealed DNA hypomethylation of the *p15$^{INK4}$* promoter (20% versus 14%) in BMS309403-treated patient

cells (Figure S8c). These data support the idea that BMS309403 possesses DNA hypomethylating activity.

### Treatment with BMS309403 alters the behavior of leukemia cells *in vitro* and *ex vivo*

Given the contribution of FABP4 deregulation to leukemia growth, we sought to examine the response of leukemia cells to BMS309403 treatment. Exposure of AML cell lines and primary cells to BMS309403 did not significantly affect apoptosis as assessed by Annexin V-FITC staining (not shown), but greatly attenuated the clonogenic potential compared to control cells (C1498, 59% inhibition; MV4-11, 51% inhibition; Kasumi-1, 67% inhibition; patient, 44% inhibition; AE9a, 61% inhibition; Figure 5 a and b). Further, BMS309403-treated leukemia cells underwent granulocytic differentiation (Figure 5c). Because CD11b serves as a cell differentiation biomarker,[14] we assessed CD11b levels by FACS in the above BMS309403-treated cells. Data indicated that CD11b expression was significantly upregulated by BMS309403 (C1498, 6.74% versus 31.0%; THP1, 43% versus 63%; Kasumi-1, 56% versus 87%; SKNO-1, 28% versus 69%; patient, 33% versus 57%; AE9a, 45.3% versus 87.7%; Figure 5d). These results suggest that BMS309403 impairs the proliferative rate of leukemia cells by inducing cell differentiation, but not by promoting apoptotic cell death. Finally, BMS309403-induced cell differentiation could be disrupted by adding the FABP4 protein into culture medium (Figure 5e), supporting that the added FABP4 might bind and sequentially reduce the biological effects of BMS309403, a further confirmation of FABP4 specificity.

### Treatment with BMS309403 markedly suppresses *in vivo* leukemia growth

To explore the emerging roles of FABPs in leukemia therapy, we initially analyzed publicly accessible raw database GSE12417.[29] No significant association of FABP1, FABP2, FABP3, FABP5 and FABP7 upregulation with poor patient survival was observed (Figure S9). Although FABP6 levels displayed positive association with patient outcomes, the role of FABP6 in cancer is not known. Given the cell autonomous and non-autonomous roles of FABP4 in leukemia progression,[6] we mainly tested the therapeutic potential of FABP4 *in vivo*. Because C57BL/6 mice have baseline FABP4 (~20 ng/ml) in circulation and leukemia cells were affected even at 10 ng/ml of FABP4 protein,[6] these mice were given one dose of BMS309403 (5 mg/kg) intraperitoneally 12 hours prior to cell injection so that the baseline activity of FABP4 could be inhibited. The second injection was given 3 days after the intravenous injection of C1498 ($0.5 \times 10^6$) cells. After administration of 5 mg/kg three times over 3-days, an additional 10 mg/kg was given twice over 3-days. The leukemia-bearing mice injected with vehicle only were used as controls. Note that the selection of BMS309403 dosages and treatment strategy was based on evidence from previous studies.[36–38] Consistent with its inhibitory effects on AML cells *in vitro* and *ex vivo*, BMS309403 administration markedly lowered the number of WBCs (Figure S10a) and reversed splenomegaly (766 ± 82 versus 412 ± 52 mg; Figure 6a). The quantification of metastatic foci revealed that the mice treated with BMS309403 exhibited fewer nodules and smaller lesion areas in lungs (16.7 ± 4.7 versus 6.1 ± 2.2) and livers (19.7 ± 3.8 versus 2.0 ± 1.0) (Figure 6 b and c). In addition, H&E stained sections of lungs and livers displayed less infiltration of blasts into these organs (Figure 6d) compared with vehicle control, indicating less aggressive leukemia growth in lungs and livers. The reduction of Ki-67 positive cells

argued for anti-proliferative effects of BMS309403 on leukemia cells (Figure S10b). Moreover, BMS309403 therapy efficiently impaired BM clonogenic potential (Figure S10c) and rescued granulocytic differentiation of myeloid cells (Figure 6e). In fact, the majority of BM cells from BMS309403-treated mice were post-mitotic cells containing metamyelocytes, bands and segmented neutrophils (73% increase; Figure 6f). BMS309403-induced cell differentiation *in vivo* was further demonstrated by the higher levels of CD11b expression in BM from BMS309403-treated than those from vehicle-treated leukemia-bearing mice (27.3% versus 3.72%; Figure S10d). Accordingly, the survival of BMS309403-treated leukemia-bearing mice was longer than that of the control group (Figure 6g).

To mimic human AML therapy, we first engrafted C57BL/6 mice (n = 8) with C1498 cells, and began BMS309403 treatment when the WBC accounts were significantly higher in leukemia cell-engrafted mice than in normal mice. Consistently, compared to the vehicle group, BMS309403 treatment greatly suppressed the development of leukemic disease, as indicated by a significant decrease of WBCs (Figure S11a) and spleen weight (655 ± 76 versus 243 ± 35 mg; Figure S11b). Giemsa–stained BM and H&E-stained sections of spleen, lung and liver from BMS309403-treated mice showed no significant infiltration of leukemic blasts, which were readily detectable in BM and organs of vehicle-treated subjects (Figure S11c). In agreement with the pathological phenotype, BMS309403-treated mice had a significantly longer survival time (Figure S11d). We did not observe obvious alterations in mouse body weight, food intake or mobility (not shown), suggesting that the side effects of BMS309403 are likely to be minimal in the current settings. Mechanistically, the antileukemic effects of BMS309403 *in vivo* were mediated by its DNA hypomethylating activities, because BM from BMS309403-treated mice had decreased *DNMT1* expression (Figure S12a), reduced global DNA methylation (Figure S12b) and upregulated methylation-silenced $p15^{INK4B}$ (Figure S12c). Thus, lowering or inhibiting the circulating FABP4 might represent a promising approach in AML management.

## Discussion

In this study, we demonstrate that DNMT1 deregulation accounts for FABP4 abnormality in AML cells, and this process appears to be mediated by VEGF signaling. We show that FABP4 is a novel druggable candidate for leukemia intervention. We present evidence showing that BMS309403, a selective FABP4 inhibitor, blocks leukemia growth *in vitro, ex vivo* and *in vivo* mechanistically by reversing aberrant DNA methylation and re-activating epigenetically-silenced $p15^{INK4B}$. Together with our recent report[6] showing that FABP4 augments AML disease progression through an epigenetic abnormality in AML cells, these findings further underscore the role of FABP4 in leukemia progression and epigenetic abnormality, elucidate a crucial and vicious FABP4-DNMT1 loop in leukemia biology (Figure S13), highlight the promise and merit of targeting the FABP4-DNMT1 loop to treat leukemia, and discover a novel type of DNA hypomethylating agent that mechanistically differs from the classical DNA methylation inhibitors.

Several recent studies have highlighted the importance of FABP4 acting as a master gene in controlling lipid metabolism and cancer cell proliferation.[6–9] Although our findings suggest a causal relationship between SFAs and FABP4 production, the molecular basis of *FABP4*

deregulation remains elusive, particularly in the context of leukemia cells. The simultaneous upregulation of both *DNMT1* and *FABP4* in obesity and leukemia cells raises the possibility that DNMT1 could reciprocally modulate the *FABP4* gene. In fact, forced *DNMT1* expression increased, whereas *DNMT1* inactivation reduced, FABP4 production. Mechanistically, DNMT1 regulates *FABP4* in a methylation-independent manner, because fewer CpGs were identified at the *FABP4* promoter. Actually, our data suggest that DNMT1 modulates *FABP4* partially through VEGF signaling, a key pathogenic factor in obesity and leukemia,[6, 24–28] because ectopic *DNMT1* expression enhanced, whereas *DNMT1* depletion inhibited, VEGF expression. Treatment with the VEGF protein significantly upregulated FABP4 and rescued the repressive effects of *DNMT1* attenuation on FABP4 expression. Given the significant contribution of FABP4 to *DNMT1* regulation,[6] we conclude that FABP4 and DNMT1 shape a positive regulatory circuit in leukemia cells, in which FABP4 regulates *DNMT1* expression through the IL-6/STAT3 axis, whereas DNMT1 controls *FABP4* by VEGF signaling (Figure S13).

While pharmaceutical approaches to target FABP4 for improved metabolic health are under investigation, no study has been initiated to exploit FABP4 as a therapeutic target in leukemia. Although many leukemia patients (≤ 20%) achieve remission with initial therapies, including decitabine and azacitidine, most patients, particularly elderly adults, are not cured. Accumulating evidence indicates that *DNMT1* gene abundance is essential to leukemia progression, but few agents target the key regulators of the *DNMT1* gene, particularly from a metabolic standpoint. The isocitrate dehydrogenase (IDH) is a metabolic enzyme that is mutated in multiple cancers.[39] IDH mutations alter genomic DNA methylation, thereby serving as potential drug targets. In fact, an inhibitor for IDH mutation has been developed and shown anticancer activity against glioma and leukemia,[40, 41] but it acts independently of DNA methylation.[40] Because FABP4 is a molecular linker of irregular lipid metabolism and aggressive leukemia by virtue of altered DNA methylation, pharmacological targeting of the FABP4-DNMT1 loop represents a viable strategy to accomplish DNA hypomethylation. Indeed, treatment with BMS309403 suppressed DNMT1 expression, reduced global DNA methylation and reactivated $p15^{INK4B}$ in AML cell lines and primary cells, thus leading to disruption of cellular clonogenic frequencies and induction of cell differentiation. Importantly, BMS309403 administration in leukemia-bearing mice decreased leukemic burden and prolonged survival duration, which mechanistically took place through the BMS309403-restored DNA hypomethylation content in AML cells. Notably, this is a proof of principle study to further support how the FABP4-DNMT1 loop is important to leukemia, and to address whether pharmacological intervention of a lipid-relevant molecule could restore the perturbed epigenetic landscapes that are the hallmarks of cancers. The dosages and schedule used for BMS309403 administration require further optimization before clinical application. Overall, our studies bridge a knowledge gap concerning the causes of AML progression, reveal the pivotal importance of the FABP4-DNMT1 regulatory loop in AML pathogenesis and establish FABP4 inactivation as distinct therapeutic approach to treat leukemia and potentially, other types of cancer, mechanistically by reversing epigenetic deregulations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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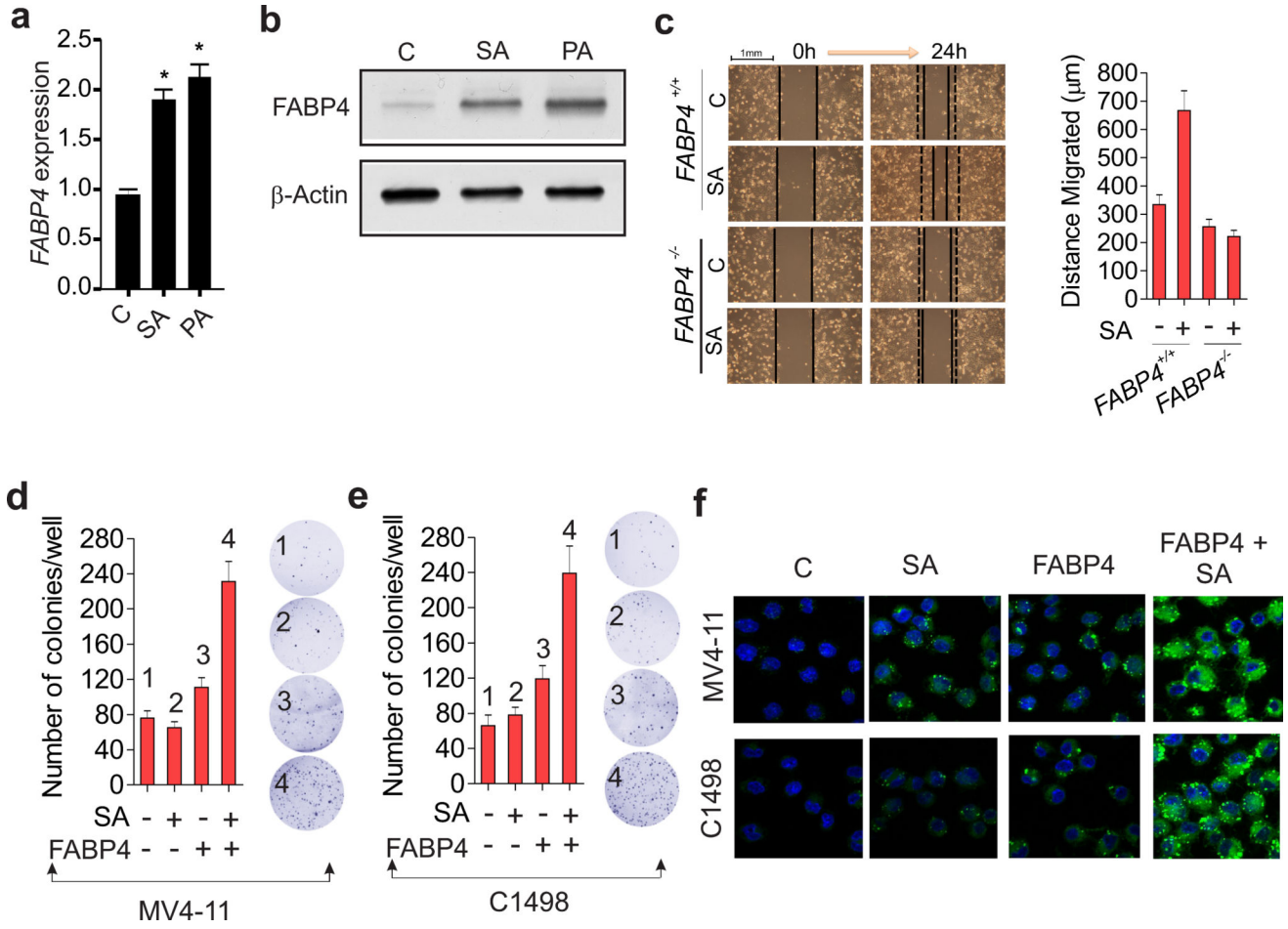


**Figure 1.**

FABP4 interacts with SFAs to promote leukemia growth. (**a** and **b**) BM cells were isolated from C57BL/6 mice, treated with the indicated fatty acids at 50 μM for 12 hours and subjected to qPCR (**a**) or Western blotting (**b**) to measure the changes in *FABP4*. $*P < 0.05$. (**c**) *FABP4*$^{+/+}$ or *FABP4*$^{-/-}$ macrophages were treated with steric acid (50 μM). When the cells grew to confluence, a wound was made and the wound width was measured at 0 or 24 hours. Left: a representative visual analysis of wound-scratch healing assays; right: the migration distance from three biological replicates. (**d** and **e**) MV4-11 (**d**) or C1498 (**e**) cells were treated with FABP4 or/and stearic acid for 6 hours and subjected to colony-forming assays. Right: a representative visual analysis of colony assays; left: graph indicates the colony number from three biological replicates, mean ± SD. For **c**–**e**, data are shown as mean values ± S.D. (**f**) Lipid droplet formation in MV4-11 and C1498 cells treated with FABP4 or/and stearic acid for 12 hours was analyzed by BODIPY staining (green) and visualized by confocal microscopy. C, control; SA, stearic acid; PA, palmitic acid.

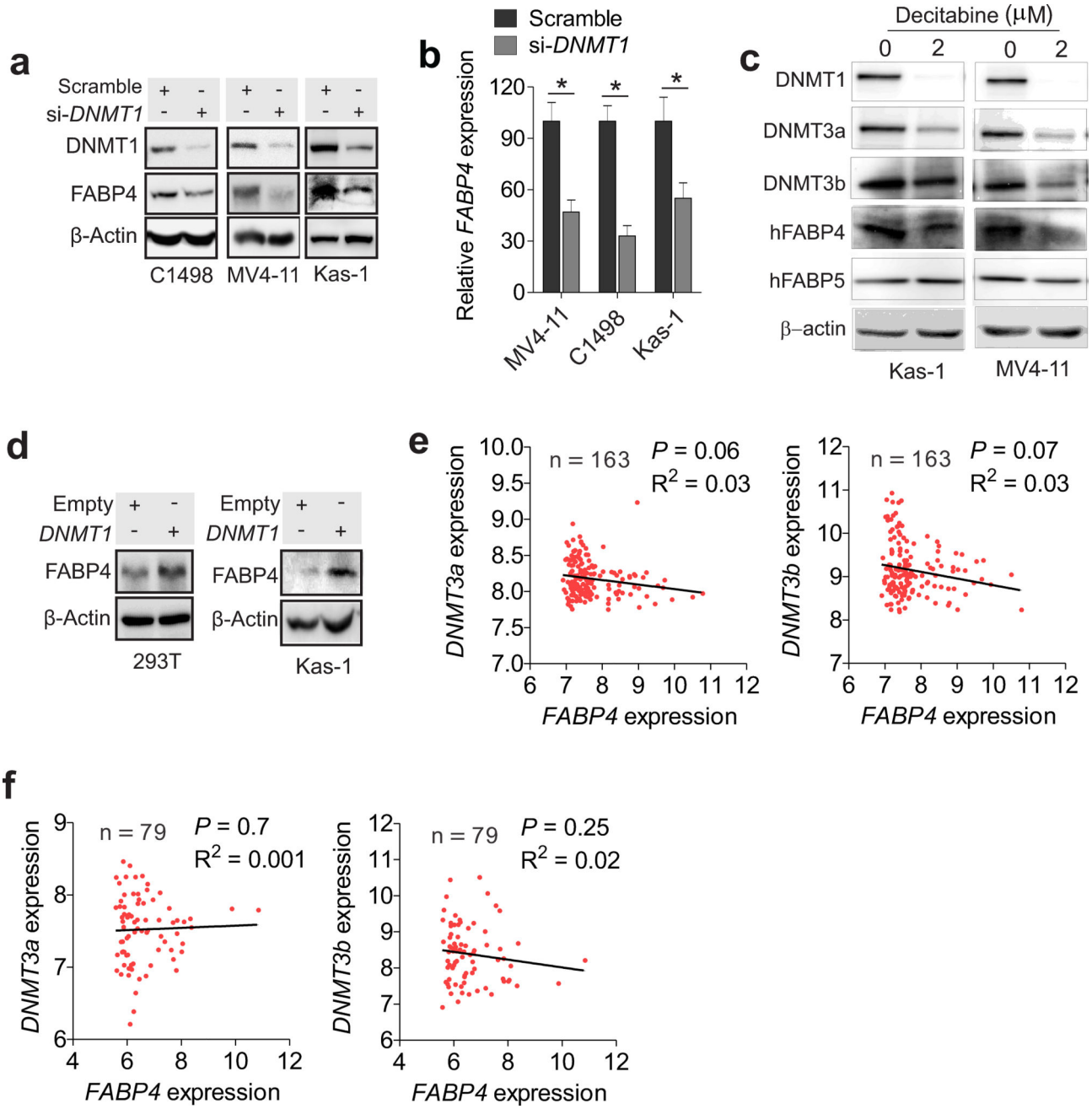


**Figure 2.**

DNMT1 positively regulates VEGF and FABP4 expression. (**a** and **b**) C1498, MV4-11 or Kasumi-1 cells were transfected with *DNMT1* siRNA and subjected to Western blotting (**a**) or qPCR (**b**). (**c**) Western blotting in MV4-11 or Kasumi-1 cells treated with 2 μM decitabine for 72 hours. (**d**) 293T or Kasumi-1 cells were transfected with *DNMT1* expression vectors and subjected to Western blotting. (**e** and **f**) Spearman correlation analysis for the mRNA expression of *FABP4* and *DNMT3a* or *DNMT3b* in AML patients (GSE12417; GPL570, **e**; GPL96, **f**). The Y or X-axis represents the fold change in

maximum expression. For Western blotting and qPCR, the experiments were performed 3 times independently; Data are shown as mean values ± SD, *$P < 0.05$, **$P < 0.01$.

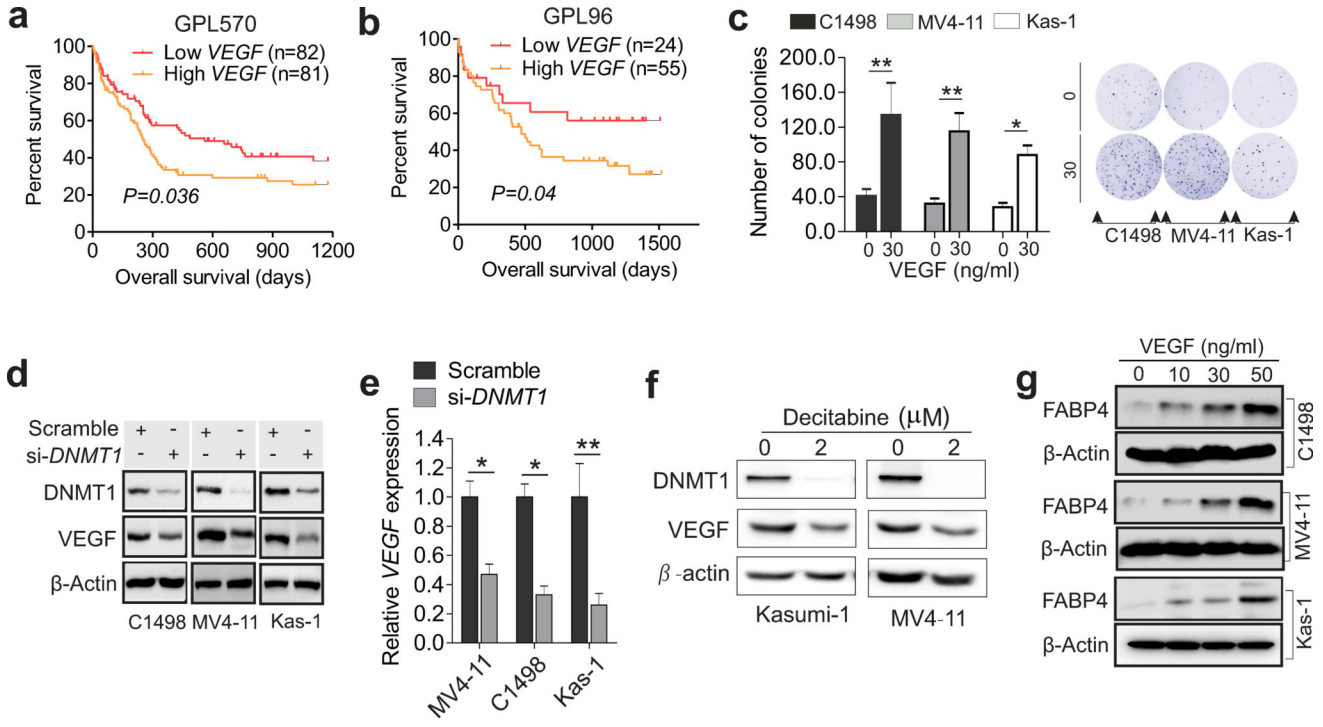


**Figure 3.**

VEGF mediates DNMT1-associated FABP4 overexpression. (**a** and **b**) Kaplan-Meier estimate for overall survival in the whole population of AML patients (GSE12417), in which high *VEGF* was compared to low *VEGF* (log-rank test). (**c**) Colony-forming assays for C1498, MV4-11 and Kasumi-1 cells treated with the VEGF protein. Graph shows the colony number from 3 independent experiments. (**d** and **e**) Western blotting (**d**) or qPCR (**e**) of C1498, MV4-11 and Kasumi-1 cells transfected with *DNMT1* siRNA. (**f**) Western blotting of Kasumi-1 and MV4-11 cells treated with 2 μM decitabine for 72 hours. (**g**) Western blotting of C1498, MV4-11 and Kasumi-1 cells treated with VEGF recombinant protein (30 ng/ml). For Western blotting and qPCR analyses, the experiments were performed 3 times independently; Data are shown as mean values ± SD, **P* < 0.05, ***P* < 0.01; All treatments are 48 hours unless otherwise indicated; si, siRNA; Kas-1, Kasumi-1.

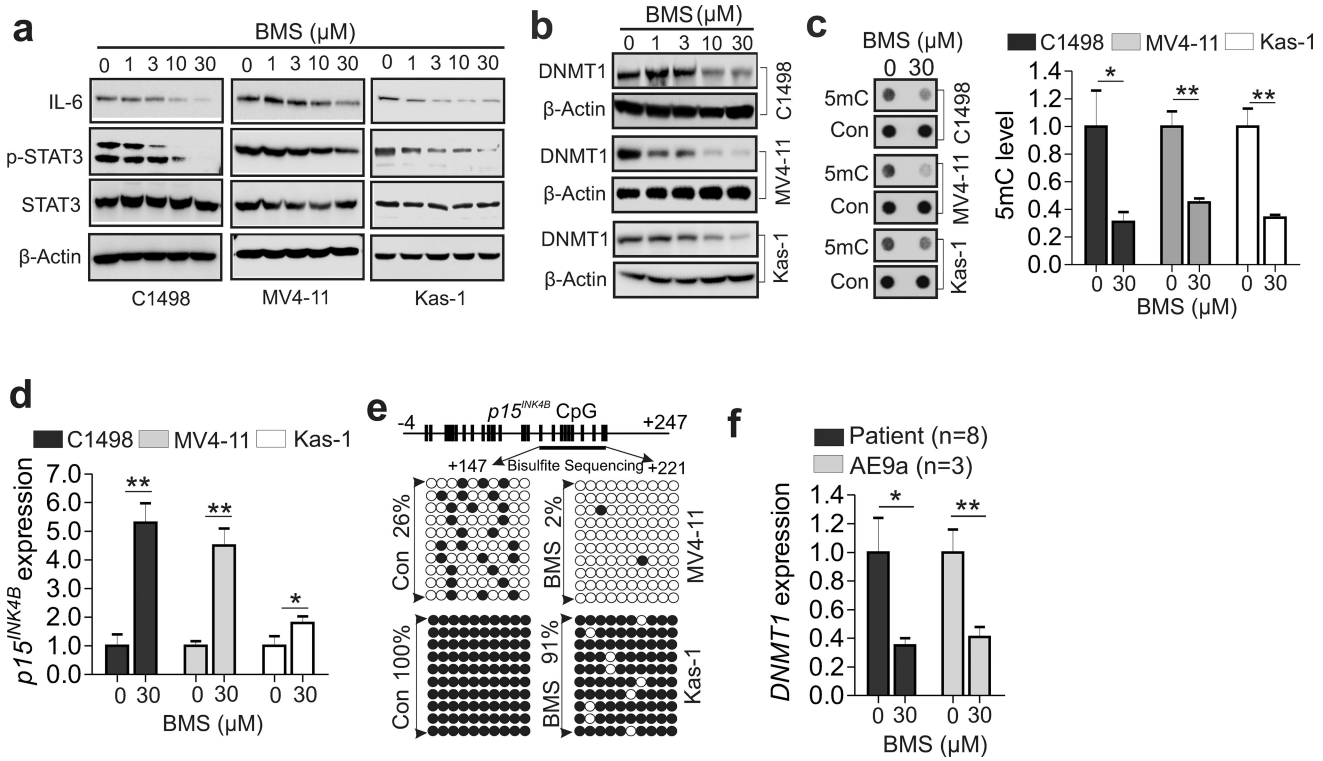


**Figure 4.**

Pharmacological inhibition of FABP4 induces DNA hypomethylation and restores the epigenetically silenced *p15*$^{INK4N}$. (**a** and **b**) Western blotting of C1498, MV4-11 or Kasumi-1 cells exposed to BMS. (**c**) C1498, MV4-11 or Kasumi-1 cells were treated with BMS and the genomic DNA was subjected to Dotblotting. Left, representative images of dots; right, graph shows quantification of dot intensity. (**d**) qPCR of C1498, MV4-11 or Kasumi-1 cells treated with BMS. (**e**) Bisulfite sequencing of the *p15*$^{INK4B}$ promoter in MV4-11 or Kasumi-1 cells treated with BMS. Results of 10 clones are presented. Vertical bars indicate CpG locations, arrows indicate the bisulfite sequencing region, open circles show unmethylated CpG, and solid circles show methylated CpGs. (**f**) qPCR of human patient (n = 8) or mouse (n = 3) AML primary cells treated with BMS. The experiments were performed three times independently and data are shown as mean values ± SD, **P* < 0.05, ***P* < 0.01. All treatments are 48 hours unless otherwise indicated; Con, control; BMS, BMS309403; Kas-1, Kasumi-1.

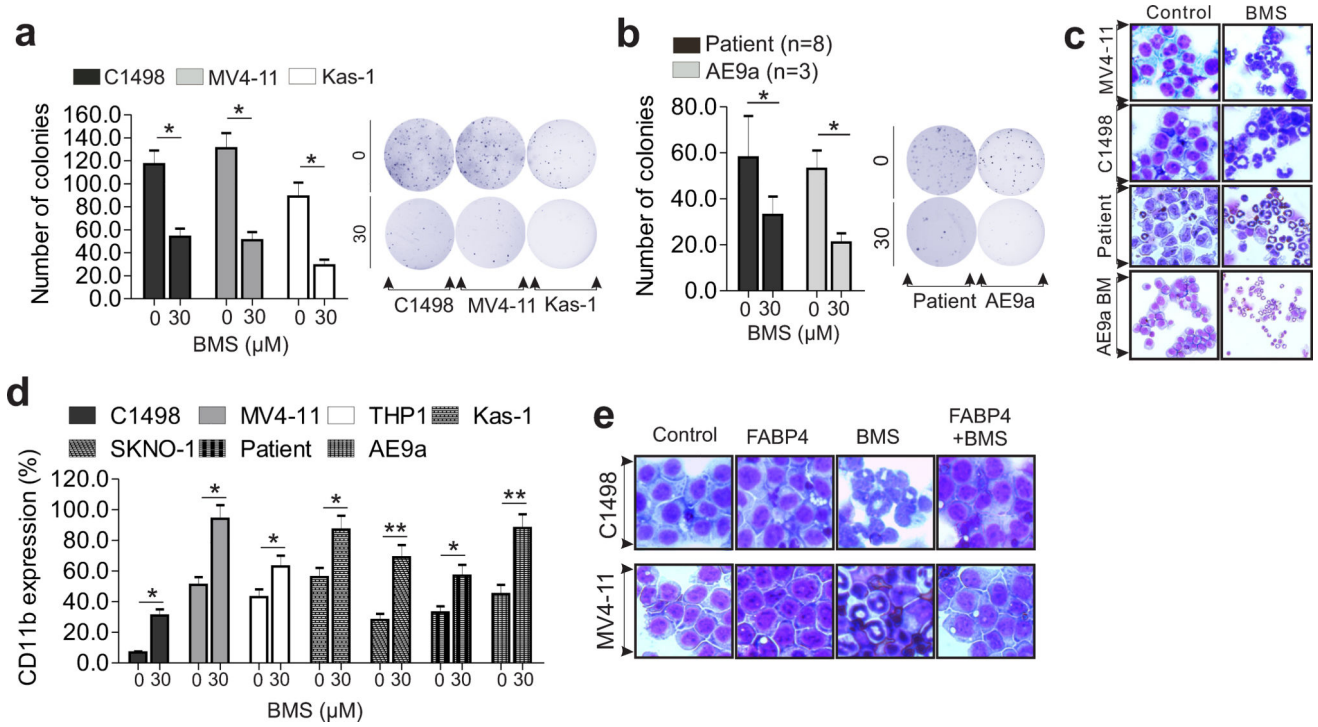


**Figure 5.**

FABP4 blockade by its inhibitor impairs leukemia growth *in vitro* and *ex vivo*. (**a** and **b**) Colony-forming assays of C1498, MV4-11 and Kasumi-1 (**a**) or human patient (n = 8) and mouse (n = 3) AML primary cells (**b**) treated with BMS. Right, graphs show quantification of colony number; left, representative images of colonies. (**c**) Visual analysis of representative images of Wright-Giemsa-stained cytospins of MV4-11, C1498 or human patient and mouse AML primary cells treated with BMS (30 μM) for 96 hours (original magnification ×400). (**d**) Graphs show quantification of CD11b+ expression in AML cells treated with BMS for 96 hours. (**e**) Representative images of Wright-Giemsa-stained cytospins of C1498 or MV4-11 cells treated with the mouse/human FABP4 protein (200 ng/ml) only, BMS (30 μM) only or the FABP4 protein plus BMS for 96 hours (magnification × 400). The experiments were performed three times independently and data are shown as mean values ± SD, *$P < 0.05$, **$P < 0.01$; Kas-1, Kasumi-1.

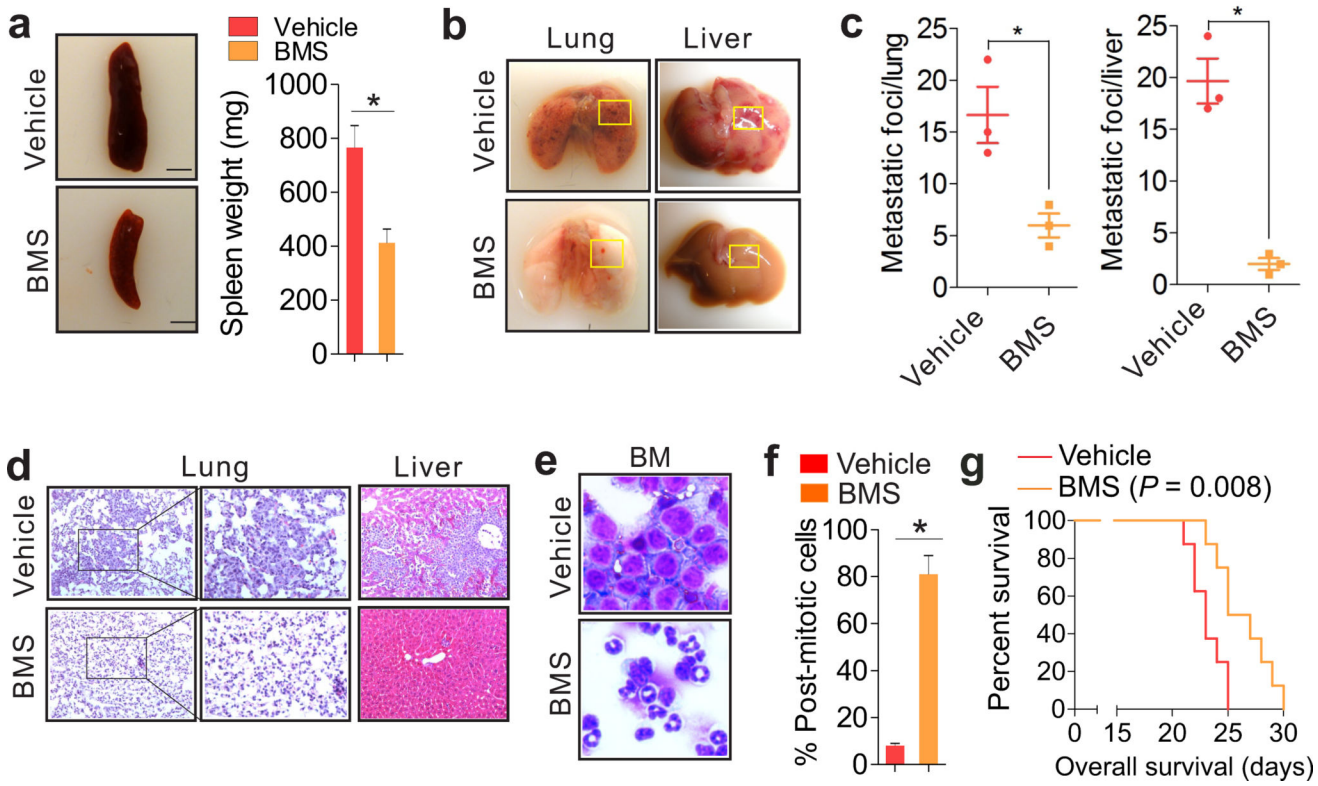


**Figure 6.**

FABP4 inactivation *in vivo* achieves leukemia remission. C1498 cells ($0.5 \times 10^6$) were intravenously injected into 4–6 weeks old C57BL/6 mice. Twelve hours prior to cell injection, a BMS (5 mg/kg) was initiated by a one-time dose. After an additional 3 doses of BMS were administered, another 10 mg/kg was given twice. (**a**) Photos show representative visual analysis and the graph indicates the calculated weight of spleens (scale bars, 5.0 mm). (**b**) External views of lungs and livers. (**c**) Graphs show quantifications of tumor nodules growing on lungs or livers. (**d**) H&E staining of lung or liver sections (original magnification ×200, ×400) from boxed areas in **b**. (**e**) Wright-Giemsa-stained BM (original magnification ×400). (**f**) Graph shows quantification of post-mitotic cells from (**e**). (**g**) Effects of BMS on survival of leukemia-bearing mice (n = 6) were determined by the Kaplan-Meier estimate (log-rank test). Note, the survival time is from the start of leukemia cell injection. In **a**–**f**, n = 3; BMS, BMS309403. Data are shown as mean values ± SD, $*P < 0.05$.