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Small-molecule Hedgehog inhibitor attenuates the leukemia-initiation potential of acute myeloid leukemia cells

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Key words

Acute myeloid leukemia, Hedgehog, leukemia stem cells, self-renewal, Smoothened

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Aberrant activation of the Hedgehog signaling pathway has been implicated in the maintenance of leukemia stem cell populations in several model systems. PF-04449913 (PF-913) is a selective, small-molecule inhibitor of Smoothened, a membrane protein that regulates the Hedgehog pathway. However, details of the proof-of-concept and mechanism of action of PF-913 following administration to patients with acute myeloid leukemia (AML) are unclear. This study examined the role of the Hedgehog signaling pathway in AML cells, and evaluated the *in vitro* and *in vivo* effects of the Smoothened inhibitor PF-913. In primary AML cells, activation of the Hedgehog signaling pathway was more pronounced in CD34⁺ cells than CD34[−] cells. *In vitro* treatment with PF-913 induced a decrease in the quiescent cell population accompanied by minimal cell death. *In vivo* treatment with PF-913 attenuated the leukemia-initiation potential of AML cells in a serial transplantation mouse model, while limiting reduction of tumor burden in a primary xenotransplant system. Comprehensive gene set enrichment analysis revealed that PF-913 modulated self-renewal signatures and cell cycle progression. Furthermore, PF-913 sensitized AML cells to cytosine arabinoside, and abrogated resistance to cytosine arabinoside in AML cells cocultured with HS-5 stromal cells. These findings imply that pharmacologic inhibition of Hedgehog signaling attenuates the leukemia-initiation potential, and also enhanced AML therapy by sensitizing dormant leukemia stem cells to chemotherapy and overcoming resistance in the bone marrow microenvironment.

The Hedgehog (Hh) signaling pathway has a key role in the development and homeostasis of many organs and tissues, and aberrant activation of the Hh pathway has been linked to tumorigenesis in a variety of cancers.^(1,2) Hedgehog ligands activate the transmembrane receptor Patched (PTCH), which induces a conformational change in Smoothened (SMO), a G protein-coupled cell receptor that regulates the Hh pathway.⁽³⁾ The conformational change in SMO enables its activation and triggers downstream signaling, with subsequent induction of the glioma-associated oncoprotein (GLI) transcription factors GLI1, GLI2, and GLI3 and transcription of target genes. Interestingly, mRNA expression of Hh signaling pathway components has been reported in acute myeloid leukemia (AML) cells.^(4,5)

The development of innovative therapies for AML that eliminate leukemia stem cells (LSCs) by targeting their specific properties represents an unmet clinical need.^(1,5–8) However, specific targeting to eradicate LSCs, which may be essential to prevent disease recurrence and achieve permanent remission, is a highly challenging task. The Hh signaling pathway is required for maintenance of the LSC population in several experimental systems.^(5,9,10) In the bone marrow

microenvironment, stromal cells are a rich source of cytokines, and some secreted cytokines suppress the induction of cell death in myeloid leukemia cells exposed to chemotherapy. However, the contribution of Hh signaling components in the leukemia microenvironment remains poorly understood.

PF-04449913 (PF-913) is a small-molecule drug that selectively binds and inhibits SMO.^(2,11) In patients with hematologic malignancies, including AML, treatment with PF-913 was safe and well-tolerated.⁽¹¹⁾ However, the mechanism of action of PF-913 and biomarkers that reflect successful AML therapy are unclear.

In this study, the relevance of the Hh signaling pathway to AML cells was examined, and evidence of the proof-of-concept and mode of action of the SMO inhibitor was found. Furthermore, pharmacologic inhibition of Hh signaling with PF-913 attenuated the leukemia-initiation potential of AML cells.

Materials and Methods

Cell lines and primary AML cells. Acute myeloid leukemia cell lines were cultured in RPMI-1640 medium (Sigma, St.

Louis, MO, USA) containing 10% FBS (Gibco-BRL, Grand Island, NY, USA), and HS-5 cells were cultured in DMEM (Sigma) containing 10% FBS. Human leukemic blasts were obtained from patients with AML, all of whom provided written informed consent. The study was carried out in accordance with the Declaration of Helsinki and approved by the institutional ethics committee of Nagoya University (Nagoya, Japan). For *in vitro* experiments, primary AML cells were cultured in RPMI-1640 medium containing 10% FBS.

Reagents. PF-913 was supplied by Pfizer (La Jolla, CA, USA). For *in vitro* experiments, PF-913 was stored as a 10^{-2} M stock solution in DMSO. For *in vivo* experiments, PF-913 was formulated as a 10 mg/mL solution in 0.5% methylcellulose (Sigma) as the vehicle. For *in vitro* experiments, cytosine arabinoside (Ara-C; Sigma) was stored as a 10^{-2} M stock solution in PBS. For *in vivo* experiments, Ara-C was formulated into a 10 mg/mL solution in PBS vehicle. The recombinant N-terminal portion of human sonic Hedgehog (SHH; R&D Systems, Minneapolis, MN, USA) was used at a concentration of 0.5 μ g/mL.

Immunoblotting. Antibodies against SMO were purchased from Abcam (Cambridge, UK). Antibodies against β -actin were from Cell Signaling Technology (Boston, MA, USA). Immunoblotting was carried out according to standard protocols as previously described.^(12,13)

Flow cytometry. Primary AML cells from patients were stained with anti-CD34-APC and anti-CD38-PE-Cy7 antibodies (1:100; Becton Dickinson, San Jose, CA, USA) for 30 min on ice, and labeled with DAPI. The DAPI-negative cells were sorted for CD34 and CD38 expression using FACS (FACSARIA; Becton Dickinson). Cells were acquired by FACSARIA and analyzed with FlowJo software (Ashland, OR, USA). Staining of cells with Hoechst 33342 (Sigma) and Pyronin-Y (Polysciences, Warrington, PA, USA) was undertaken as previously described.⁽¹⁴⁾ Briefly, drug-treated cells were washed in Hanks staining buffer containing 1 \times HBSS (Invitrogen), 20 mM HEPES at pH 7.9, and 2% FBS, and then incubated in Hanks staining buffer containing 5 μ g/mL Hoechst 33342, at a density of 1 million cells/mL at 37°C for 45 min. Pyronin-Y was added to a final concentration of 1 μ g/mL, and the cells were incubated again for 45 min at 37°C, then washed and resuspended in Hanks staining buffer. Flow cytometry was undertaken using FACSARIA. Cells were labeled with annexin-V-FITC and DAPI after 48 h of treatment with PF-913 according to the manufacturer's protocol (Annexin-V-FLUOS Staining Kit; Roche Diagnostics, Indianapolis, IN, USA).

Real-time PCR. Total RNA was purified using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany), and reverse transcription was carried out with a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time RT-PCR was carried out according to standard procedures, using TaqMan Universal PCR Master Mix with quantitative PCR primers for GLI1 (Hs01110766_m1), GLI2 (Hs01119974_m1), GLI3 (Hs00609233_m1), PTCH1 (Hs00181117_m1), TaqMan Endogenous Control Eukaryotic 18S rRNA, and the ABI Prism 7000 Sequence Detection System. All of these reagents, primers, and equipment were from Applied Biosystems (Foster City, CA, USA). Results were normalized against 18S rRNA expression. The relative levels of mRNA were calculated using the $2^{-(\Delta\Delta C_T)}$ method.

Mouse models. Xenograft models were established in NOD/SCID/IL2r γ^{null} (NOG) mice, as previously described.^(14,15)

NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and Clea Japan (Tokyo, Japan). Briefly, AML cells (2×10^6) were i.v. transplanted into 7-week-old male NOG mice. Engraftment was confirmed at 4 and 8 weeks by detection of human CD45-positive cells in peripheral blood. After engraftment, PF-913 (100 mg/kg) or vehicle was administered at a volume of 10 mL/kg by gavage, twice daily for 10 days. Bone marrow, spleen, and peripheral blood cells were stained with anti-human CD45-PE and anti-mouse CD45-PerCP to analyze chimerism. Bone marrow cells (1×10^6) were serially xenotransplanted into NOG mice and, after 4 weeks, bone marrow, spleen, and peripheral blood cells were stained with anti-human CD45-PE and anti-mouse CD45-PerCP to analyze chimerism. All protocols were approved by the Animal Ethics Committee of Nagoya University.

Colony formation analysis. Human mononuclear cells isolated from AML patients were thawed, washed with ISCOV (Sigma) containing 2% FBS, and leukemic colony formation assays were carried out. Leukemia cells (2×10^6 to 1×10^7) were added at a dilution of 1:10 to the methylcellulose medium MethoCult H4534 (StemCell Technologies, Vancouver, Canada). The treatment drug and control DMSO were added at their indicated final concentrations, and 400 μ L MethoCult was dispensed in duplicate into the 24-well plates. Cells were incubated in a humidified incubator at 37°C with 5% CO₂, and colonies were scored manually after 14 days. Human cord blood (CB) cells were collected after full-term deliveries. Written informed consent was obtained from all participants, and the protocol was carried out in accordance with the Declaration of Helsinki and approved by the Review Board of Tokai Cord Blood Bank (Seto, Japan). Mononuclear cells were harvested by Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation. Cord blood mononuclear cells (5×10^4) were plated in the complete methylcellulose medium MethoCult (H4435; Stem Cell Technologies) with PF-913. After 14 days of culture, erythroid burst-forming units, colony-forming unit–granulocyte macrophages, and colony-forming unit–granulocyte, erythrocyte, monocyte/macrophage, and megakaryocyte colonies were counted.

DNA microarray and data analysis. Bone marrow cells from PF-913-treated mice ($n = 3$) and vehicle-treated mice ($n = 3$) were separated with the MACS human CD45 MicroBeads Kit (Miltenyi Biotec, Tokyo, Japan) into AML cells, and total RNA was purified. DNA microarray analysis was undertaken by Filgen (Nagoya, Japan) using a CodeLink Human Whole Genome Bioarray (Applied Microarrays, Tempe, AZ, USA). The Gene Set Enrichment Analysis (GSEA) algorithm was used to evaluate the statistical significance of gene expression. Gene Set Enrichment Analysis is supported by the Broad Institute (<http://www.broadinstitute.org/gsea/index.jsp>).

Statistical analysis. Data are presented as the means \pm SEM. The statistical significance of differences between groups was determined using Student's *t*-test. Comparisons among more than two groups were analyzed with the Tukey–Kramer test followed by one-way ANOVA. Statistical analyses were carried out with JMP pro software (SAS Institute, Cary, NC, USA).

Results

Leukemia-initiation potential attenuated by Hh signaling inhibitor. Investigation of the *in vivo* effects of PF-913 treatment in NOG mice, the first transplant recipients (Fig. 1a),

indicated that tumor burden was not reduced (Fig. 1b, top panels). In a serial transplantation mouse model, PF-913 treatment attenuated the leukemia-initiation potential of AML cells (Fig. 1b, bottom panels). Furthermore, using another primary AML cell sample in a serial transplantation mouse model, PF-913 eliminated the self-propagation capacity of AML cells (Fig. 1c). Following *in vivo* treatment with PF-913, human CD45⁺ cells were harvested from the bone marrow of recipient NOG mice for DNA microarray assays. Gene set enrichment analysis revealed that the self-renewal signature associated with epithelial cancer stem cells and the cell cycle regulation signature were strongly correlated with target genes during PF-913 treatment (Figs. 2, S1).^(16,17)

Cell cycle status of quiescent AML cells altered by Hh signaling inhibitor. Annexin-V/DAPI staining of primary AML cell cultures revealed that *in vitro* treatment with PF-913 for 48 h did not induce substantial cell death (Fig. 3a). Incubation of primary AML cells with PF-913 attenuated SMO-targeting gene

transcripts that were activated by the addition of SHH (Fig. S2b), and reduced the fraction of CD34⁺CD38[−] cells (Fig. 3b). In human CD45⁺ cells derived from leukemic mouse bone marrow, the percentage of G₀ cells was reduced after 48 h of incubation with PF-913 (Fig. 3c). The frequency of G₀ cells (Hoechst-33343^{low}/Pyronin-Y^{low}) was higher in CD34⁺CD38[−] cells than in the CD34[−]CD38⁺ cell population (Fig. S3a). Protein expression of SMO was higher in CD34⁺ than CD34[−] cells (Fig. S3b), and the Hh signaling pathway was more highly activated in CD34⁺ than CD34[−] cells (Fig. S3c).

Using MARIMO cells, an AML cell line harboring abnormal complex karyotypes,⁽¹⁸⁾ PF-913 treatment induced minimal cell death accompanied by a reduction in the relative quiescent cell population (Fig. S4). We carried out comprehensive analyses of the metabolomic profile of MARIMO cells during PF-913 treatment. As a preliminary observation, PF-913 treatment induced the tendency of activation of all

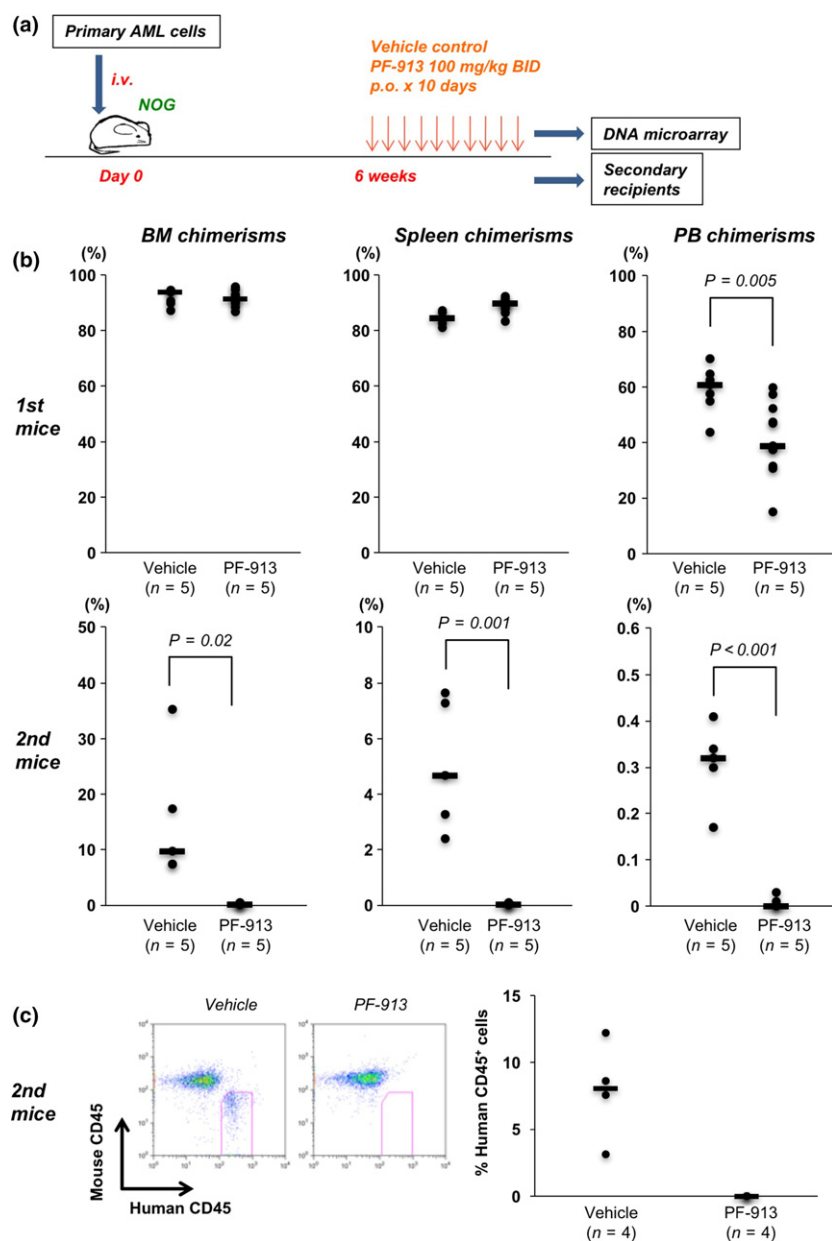


Fig. 1. *In vivo* treatment with PF-04449913 (PF-913) eliminated self-renewal capacity of acute myeloid leukemia (AML) cells. (a) Overview of experimental design regarding *in vivo* treatment with PF-913 in AML NOG mice. Mice were injected with AML cells and PF-913 (100 mg/kg) or vehicle at a volume of 10 mL/kg by gavage, twice daily for 10 days after engraftment of AML cells. Bone marrow (BM), spleen, and peripheral blood (PB) cells were stained with anti-human CD45-PE and anti-mouse CD45-PerCP to analyze chimerism. Bone marrow cells (1×10^6) were serially xenotransplanted into NOG mice; after 4 weeks, BM, spleen, and PB cells were stained with anti-human CD45-PE and anti-mouse CD45-PerCP to analyze chimerism. Following *in vivo* treatment with PF-913, human CD45⁺ cells were harvested from BM of recipient NOG mice for DNA microarray assays. (b) Efficacy of *in vivo* treatment with PF-913 in AML NOG mice line. Top panels, data in the primary (1st) transplantation mice; bottom panels, data in the serial (2nd) transplantation mice. Horizontal bars indicate the mean. (c) Percentage of AML cells in BM of the serial (2nd) transplantation mice. Left panel, representative data. Right panel, horizontal bars indicate the mean.

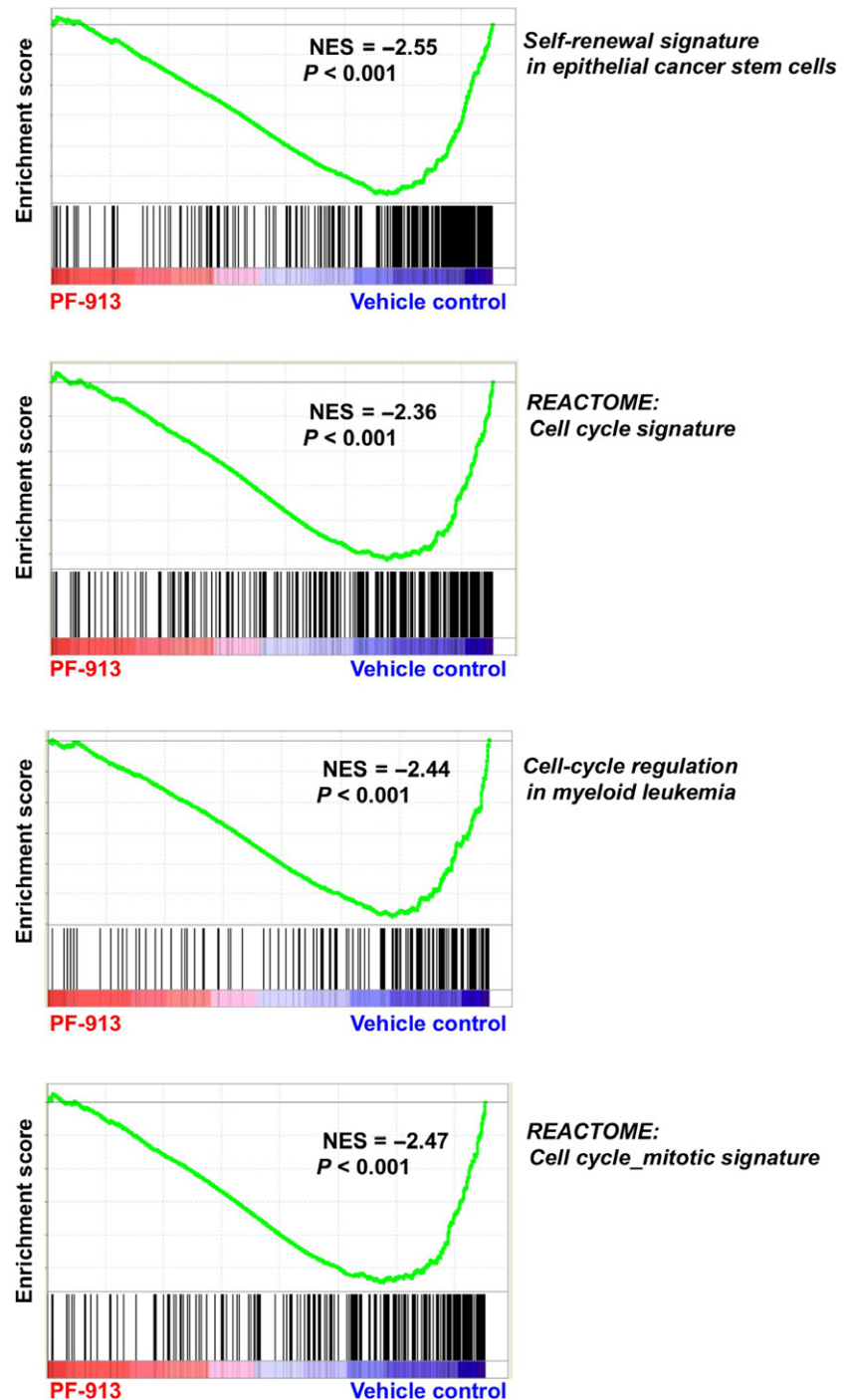


Fig. 2. Gene Set Enrichment Analysis (GSEA) plot in vehicle-treated cells versus PF-04449913 (PF-913)-treated cells. The epithelial cancer stem cells signature and the cell-cycling regulation-associated signatures were strongly correlated with target genes during PF-913 treatment. A GSEA algorithm was used to evaluate the statistical significance of gene expression. GSEA calculates normalized enrichment scores (NES), which are values assigned to each gene and are set after normalization across all analyzed gene sets.

components in the pentose-phosphate pathway (Fig. S5), which has been implicated in various stages of cancer proliferation.⁽¹⁹⁾

These data suggest that treatment with PF-913 abrogates the quiescent property of AML cells by altering the cell cycle status of dormant fractions.

PF-913 treatment spares the self-renewal capacity of normal CB cells. PF-913 inhibited clonogenic growth of primary AML cells in replating assays (Fig. 4a,b). To examine the toxicity of PF-913 in normal cells, mononuclear cells from human CB were plated in methylcellulose with PF-913, and the colonies were counted after 14 days (Fig. 4c). PF-913 had a negligible

effect on colony formation of CB mononuclear cells (Fig. 4c, d), and did not inhibit clonogenic growth of CB mononuclear cells in replating assays (Fig. S6), indicating that PF-913 treatment does not suppress the self-renewal capacity of human CB mononuclear cells.

PF-913 sensitized AML cells to Ara-C and abrogated resistance to Ara-C. The effect of the combination of PF-913 with Ara-C was investigated in AML cells cocultured with HS-5 stromal cells (Fig. 5a). HS-5 cells are known to secrete multiple cytokines, including Hh ligands.⁽²⁰⁾ In MOLM-14 cells harboring an FMS-like tyrosine kinase 3/internal tandem duplication (FLT3/ITD) mutation, SMO-targeting gene transcripts were

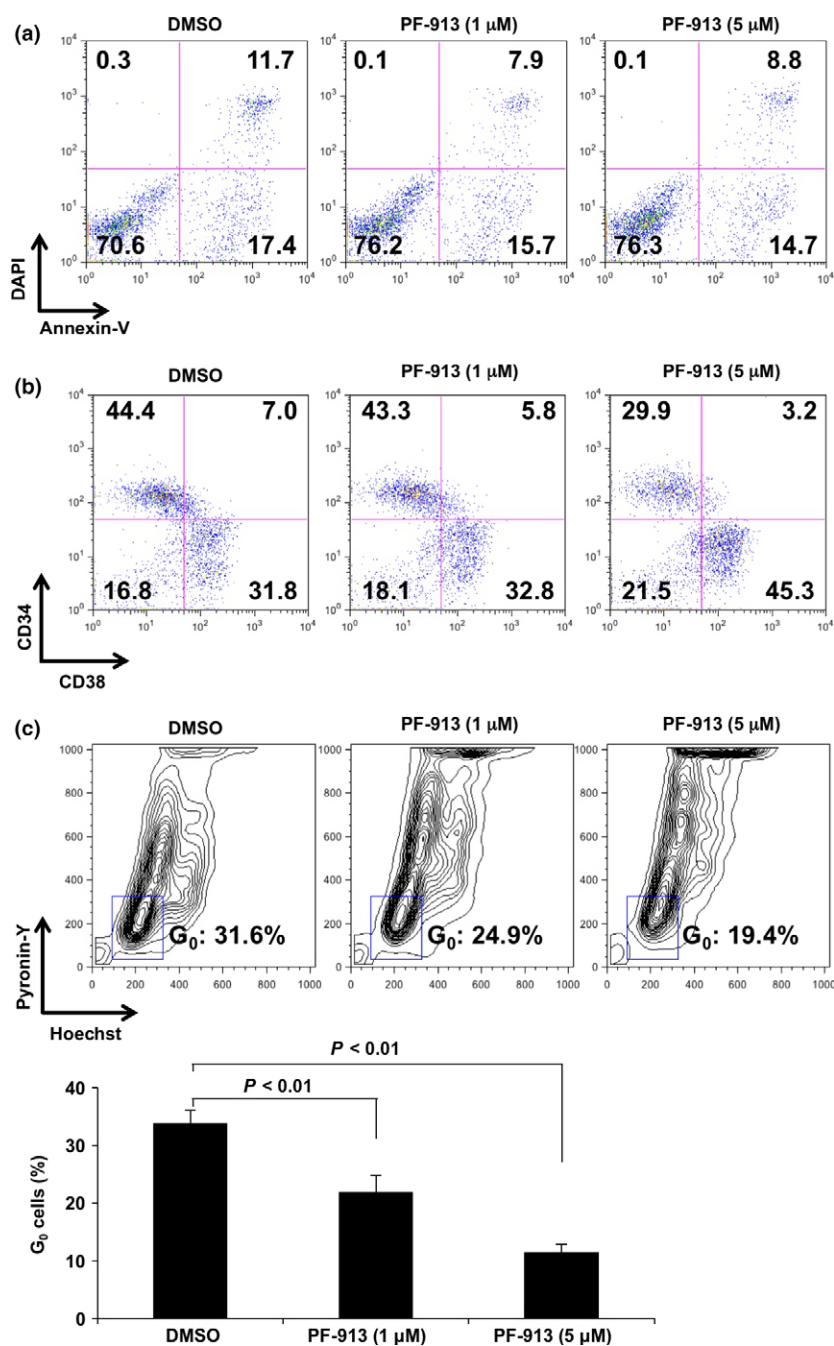


Fig. 3. *In vitro* treatment with PF-04449913 (PF-913) decreased quiescent cells. (a) Annexin-V/DAPI staining after treatment with PF-913 for 48 h showed that PF-913 treatment induced minimal cell death in primary acute myeloid leukemia cells under addition of sonic Hedgehog. Cells were acquired by FACSaria and analyzed with FlowJo software. (b) Primary acute myeloid leukemia cells were treated with PF-913 for 48 h under addition of sonic Hedgehog. DAPI-negative viable cells were gated. (c) Percentage of G₀ cells (Hoechst-33343^{low}/Pyronin-Y^{low}) after treatment with PF-913 for 48 h in human CD45⁺ cells derived from bone marrow of leukemic mice. Data are expressed as mean \pm SEM.

upregulated by coculture with HS-5 stromal cells, and down-regulated after PF-913 treatment (Figs 5b,S7).

Moreover, treatment with PF-913 abrogated resistance to Ara-C in MOLM-14 cells cocultured with HS-5 stromal cells (Fig. 5c). After addition of an SHH ligand, PF-913 treatment abrogated resistance to not only Ara-C (Fig. S8a), but also to the FLT-3-kinase inhibitor sunitinib (Fig. S8b). These data provide a rationale for combining PF-913 with conventional drugs and kinase inhibitors in AML therapy.

In MARIMO cells, both *in vitro* and *in vivo* treatment with PF-913 sensitized AML cells to Ara-C (Fig. S9). These complementary effects may be related to the drug's mode of action, with PF-913 treatment leading to the induction of dormant fractions to cell cycling status, as depicted in Figures S4 and S5.

Discussion

Our findings suggest that pharmacologic inhibition of Hh signaling can attenuate the leukemia-initiation potential of AML cells. As previously reported, mRNA expression of Hh signaling molecules was ubiquitous in various AML cells including LSCs (Fig. S10).^(4,5) Targeting factors that maintain minimal residual disease in the bone marrow niche to extract remaining LSCs and their progeny from the protected microenvironment has attracted substantial interest over the last decade.^(21,22) This study focused on the effects of an Hh signaling inhibitor on signatures of AML cells; however, paracrine mechanisms such as bone marrow niche cells should also be evaluated during treatment of AML with Hh signaling inhibitors. To better understand the effects of PF-913 treatment on the bone

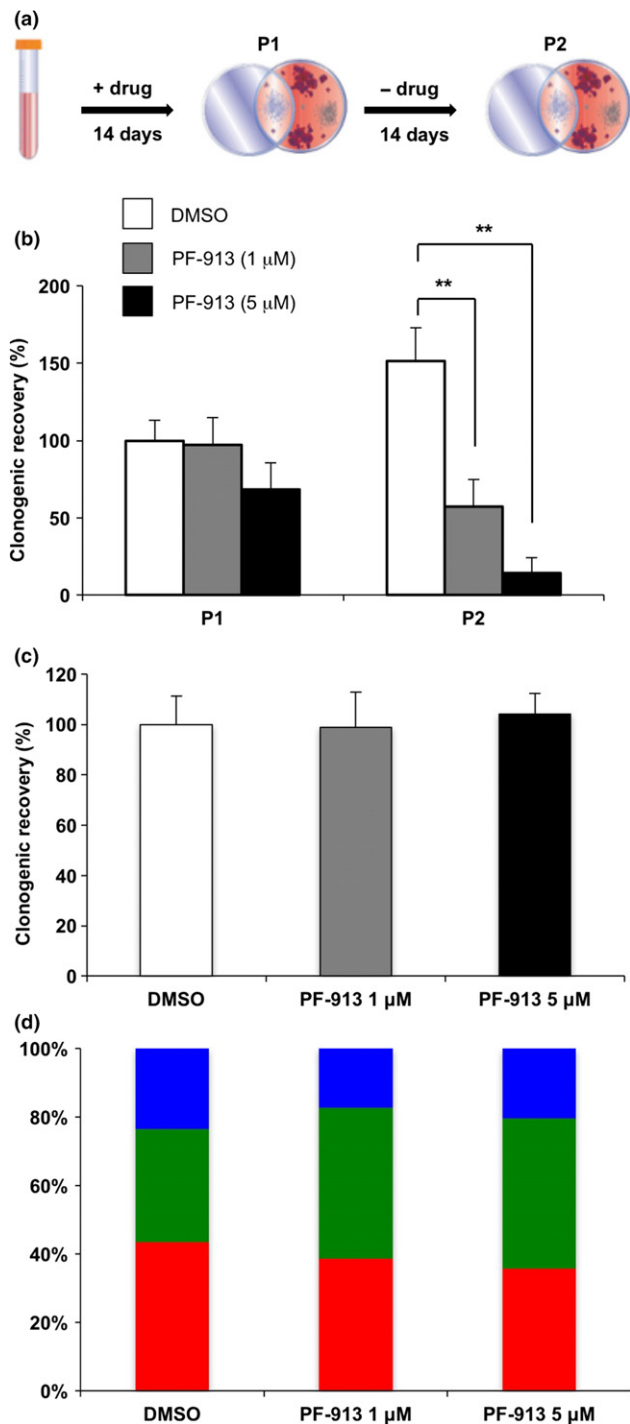


Fig. 4. PF-04449913 (PF-913) inhibited clonogenic growth of primary acute myeloid leukemia cells in the replating assay, and had a negligible effect on colony formation of cord blood (CB) mononuclear cells. (a) Acute myeloid leukemia cells were treated with PF-913 in methylcellulose, and at 14 days, colonies were counted (P1). A representative plate was then washed and cells were resuspended and replated. After an additional 14 days, colonies were counted (P2). Clonogenic recovery of untreated cells was normalized to 100%. (b) Colony formation of AML cells following treatment with PF-913 (** $P < 0.01$). Data are expressed as mean \pm SEM. (c) Mononuclear cells from human cord blood were plated in methylcellulose with PF-913. After 14 days, colonies were counted. Data are expressed as mean \pm SEM. (d) Distribution of erythroid burst-forming units (red) and colony-forming unit-granulocyte macrophages (blue), along with colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, and megakaryocyte (green) colonies.

marrow niche, a comprehensive human cell surface marker panel was used to screen for CD markers and adherent surface molecules in MARIMO cells. The screening assays showed that only expression of CD164, an adhesive glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells, which acts as a potent negative regulator of hematopoiesis, was substantially decreased after treatment with PF-913 (Fig. S11).⁽²³⁾ Non-hematopoietic cells such as stromal cells are thought to represent a more rational *in vivo* target of Hh signaling to affect AML progenitors.⁽²⁴⁾ Thus, the effects of paracrine mechanisms, including CD164 and chemokines, on stromal cells in the bone marrow microenvironment will be explored.

The present findings indicated that combined treatment with PF-913 sensitized AML cells to Ara-C, and abrogated Ara-C resistance in AML cells cocultured with HS-5 stromal cells. Ishikawa *et al.* beautifully showed resistance to Ara-C in quiescent AML stem-like cells and elimination of AML stem-like cells by induction of cell cycle entry.^(8,25) In particular, in Figures 3, S3, and S4, we showed that treatment with PF-913 directly affected the quiescent AML cells that are supposed to be refractory to monotherapy with Ara-C. Combination treatment with PF-913 and Ara-C (or a kinase inhibitor such as sunitinib) mutually compensated each weakness due to the cell cycle properties in AML cells. Recently, the SHH factor GLI1 was found to cause drug resistance through inducible glucuronidation.⁽²⁶⁾ The impact of PF-913 on this GLI1-mediated drug resistance of AML cells requires further investigation using our experimental system and samples obtained during PF-913 treatment in the clinical setting.

In this study, comprehensive GSEA revealed that Hh inhibition affects self-renewal signatures and cell cycle regulation in AML cells. It was previously reported that Hh signaling modulates cell cycle regulators in stem cells to control normal hematopoietic regeneration.⁽²⁷⁾ In the BCR-ABL-positive leukemia model, selective SMO inhibition abrogates human LSC dormancy.⁽²⁸⁾ The authors showed that treatment with PF-913 in humanized stromal cocultures reduced downstream cell cycle gene expression and sensitized BCR-ABL-positive LSCs to tyrosine kinase inhibition. A recent study also reported that inhibition of Hh signaling induced apoptosis by decreasing c-Myc protein expression in OPM1 myeloma cells.⁽²⁹⁾ By intensive scrutiny of GSEA results shown in Figure 2, we found that the decrease of c-Myc expression was common in all signatures. As shown in Figure S4, MARIMO AML cells well reflected the appearance of primary AML cells during PF-913 treatment and highly express c-Myc in the steady state, therefore, we examined c-Myc expression in MARIMO AML cells during PF-913 treatment (Fig. S12). Expression of c-Myc substantially decreased during PF-913 treatment. The downregulation of c-Myc can partly reflect the mode of action of the treatment regarding attenuating leukemia-initiation potential. Several groups have reported that two downstream effectors in the Hh pathway, GLI1 and GLI2, directly bind to the pluripotency factor, Nanog promoter, and the GLI-Nanog axis promotes stemness and growth in gliomas.⁽³⁰⁾ We also found that a change in Nanog transcripts was closely associated with GLI-target genes such as c-Myc in the immunodeficient NOG mice AML model, and Nanog transcripts can be a responsive biomarker during PF-913 therapy (data not shown). Future studies will be carried out to delineate the direct mediators, including Nanog expression and association with c-Myc, that influence self-renewal and cell cycle regulation.

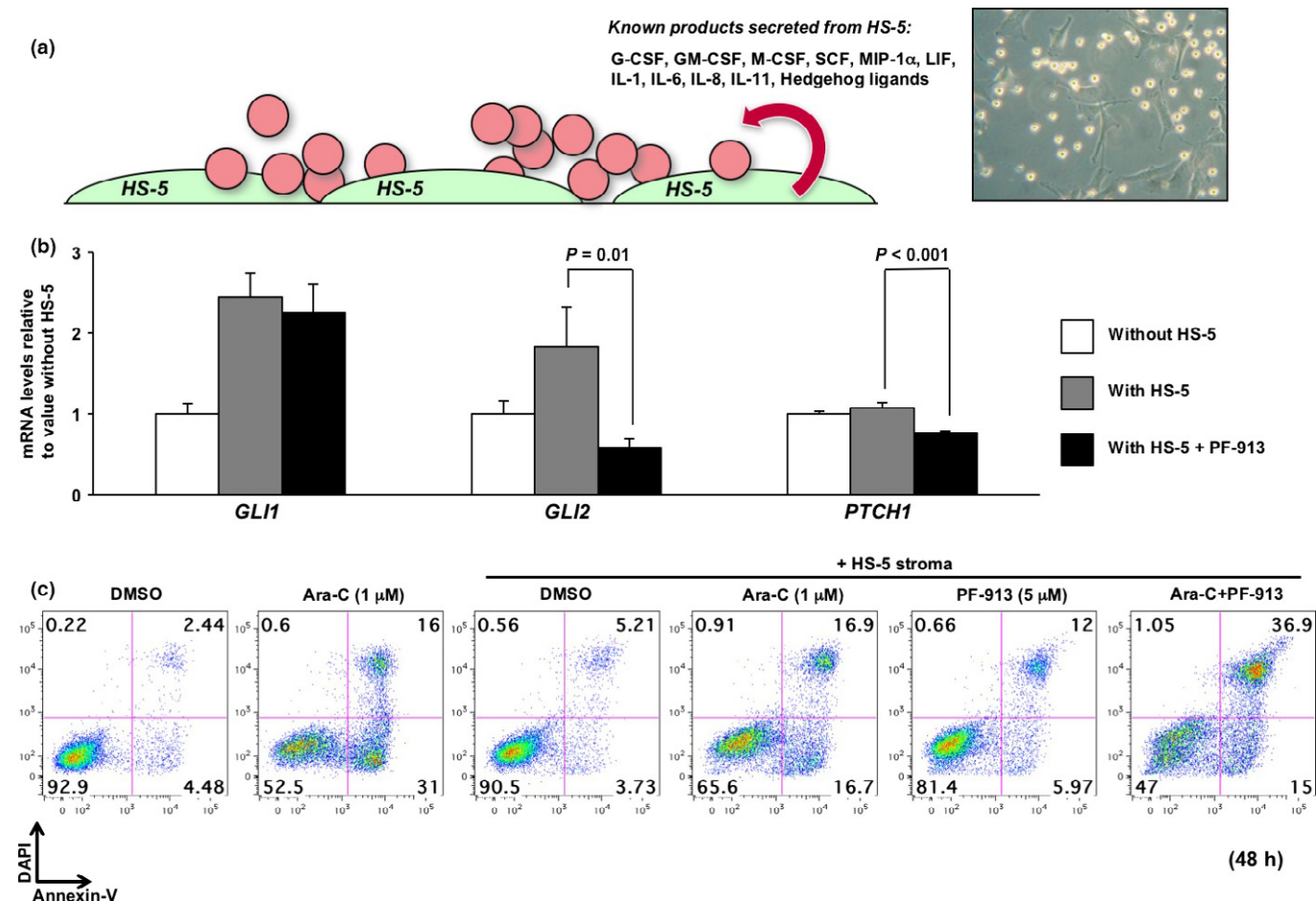


Fig. 5. PF-04449913 (PF-913) sensitized acute myeloid leukemia (AML) cells to cytosine arabinoside (Ara-C) and abrogated resistance to Ara-C. (a) AML cells cocultured with HS-5 stromal cells. HS-5 cells are known to secrete multiple cytokines, including Hedgehog ligands. The effect of the combination of PF-913 with Ara-C was investigated in AML cells cocultured with HS-5 stromal cells. (b) In MOLM-14 cells harboring an FMS-like tyrosine kinase 3/internal tandem duplication mutation, Smoothened-targeting gene transcripts were upregulated by coculture with HS-5 stromal cells, and downregulated after PF-913 treatment. mRNA levels of Smoothened-targeting genes normalized to values without HS-5. Treatment with PF-913 lasted 6 h. Data are expressed as mean \pm SEM. (c) Resistance to Ara-C by coculturing with HS-5 was abrogated by PF-913 in MOLM-14 cells. Annexin-V/DAPI staining after treatment with PF-913 (5 μ M) and Ara-C (1 μ M). Each treatment was for 48 h. Data are expressed as mean \pm SEM. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; LIF, leukemia inhibitory factor; M-CSF, macrophage colony-stimulating factor; MIP-1 α , macrophage inflammatory protein-1 α ; SCF, stem cell factor.

The indispensable role of the Hh pathway in normal hematopoiesis and leukemia is still controversial.^(7,15) Recently, expression of the Hh pathway mediator GLI was found to be a negative prognostic marker in AML.⁽³¹⁾ Lim *et al.*⁽³²⁾ also reported that Hh signaling accelerates the development of AML by enhancing signal transducer and activator of transcription 5 signaling, and the proliferation of bone marrow myeloid progenitors in AML harboring the FLT3/ITD mutation. The oncogenic role of the Hh signaling pathway in distinct types of leukemia requires further clarification.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Gene Set Enrichment Analysis plot.

Fig. S2. (a) MTT assay in primary acute myeloid leukemia cells. (b) mRNA levels of Smoothened-targeting genes.

Fig. S3. (a) Frequency of G₀ cells. (b) Expression of Smoothened (SMO) protein. (c) Expression levels of glioma-associated oncoprotein (GLI)1, GLI2, GLI3, and Patched 1 (PTCH1) transcripts.

Fig. S4. (a) Frequency of Hoechst-33343^{low}/Pyronin-Y^{low} population. (b) Annexin-V/DAPI staining. (c) MTT assay.

Fig. S5. Comprehensive analyses of the metabolomic profile.

Fig. S6. Clonogenic growth of mononuclear cells from human cord blood cells.

Fig. S7. Change of Smoothened-targeting gene transcripts.

Fig. S8. Resistance to cytosine arabinoside (Ara-C) and FMS-like tyrosine kinase-3 (FLT-3)-kinase inhibitor.

Fig. S9. Combination treatment with cytosine arabinoside (Ara-C) and PF-04449913 (PF-913).

Fig. S10. mRNA expression of Hedgehog signaling molecules.

Fig. S11. Comprehensive human cell surface marker screening.

Fig. S12. Expression of c-Myc during treatment with PF-04449913 (PF-913).