Leukemia
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ARTICLE

Acute myeloid leukemia

Histone deacetylase inhibitor targets CD123/CD47-positive cells and reverse chemoresistance phenotype in acute myeloid leukemia

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Abstract

Chemoresistance may be due to the survival of leukemia stem cells (LSCs) that are quiescent and not responsive to chemotherapy or lie on the intrinsic or acquired resistance of the specific pool of AML cells. Here, we found, among well-established LSC markers, only CD123 and CD47 are correlated with AML cell chemosensitivities across cell lines and patient samples. Further study reveals that percentages of $CD123^{+}CD47^{+}$ cells significantly increased in chemoresistant lines compared to parental cell lines. However, stemness signature genes are not significantly increased in resistant cells. Instead, gene changes are enriched in cell cycle and cell survival pathways. This suggests CD123 may serve as a biomarker for chemoresistance, but not stemness of AML cells. We further investigated the role of epigenetic factors in regulating the survival of chemoresistant leukemia cells. Epigenetic drugs, especially histone deacetylase inhibitors (HDACis), effectively induced apoptosis of chemoresistant cells. Furthermore, HDACi Romidepsin largely reversed gene expression profile of resistant cells and efficiently targeted and removed chemoresistant leukemia blasts in xenograft AML mouse model. More interestingly, Romidepsin preferentially targets $CD123^{+}$ cells, while chemotherapy drug Ara-C mainly targeted fast-growing, $CD123^{-}$ cells. Therefore, Romidepsin alone or in combination with Ara-C may be a potential treatment strategy for chemoresistant patients.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disease characterized by the proliferation and accumulation of myeloid blast cells in the bone marrow which eventually lead to hematopoietic failure. Cytarabine (Ara-C) has been the standard chemotherapy agent of induction therapy and consolidation therapy for AML since the 1960s. Despite significant progression in the treatment of newly diagnosed AML, a substantial percentage of patients do not achieve remission with standard chemotherapy, and over 50% of first complete remission patients are expected to relapse within 3 years [1]. The optimum strategy for patients with the resistant disease remains uncertain. Evidence from

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xenograft and human trials indicates that a subset of cancer cells is selectively enriched after chemotherapy. This population of cells possesses aberrant transcriptional and epigenetic programs that endow them with stemness properties. Such cells, functionally defined as cancer stem cells (CSCs), are responsible for cancer recurrence, metastasis, and chemoresistance [2–5]. Multiple LSC surface markers have been identified in patient samples and cell lines [6]. However, it remains to be determined whether subpopulations with different LSC markers have similar or varied chemoresistant abilities. A better identification and characterization of these CSC markers should provide a more comprehensive understanding of tumor developmental biology and have immediate and important clinical implications.

Epigenetic modulation is a reversible chemical modification on histones or DNA, whereby chromatin structure is manipulated to open or close regions of genome, therefore promoting or inhibiting gene transcription, respectively. The aberrant recruitment or overexpression of epigenetic modifiers has been implicated in many cancers by causing altered gene expression profile to allow for increased survival, evasion of cell death, and drug resistance [7–10]. Increasing evidence supports the hypothesis that epigenetic changes are not only a driving force behind the acquisition of drug resistance [11–13], but also contribute to survival and self-renewal of cancer stem cell [14–18].

In this study, we demonstrated leukemia stem cell (LSC) surface markers CD123 and CD47 are positively correlated to AML chemoresistance. Therefore, these two markers may serve as chemoresistant and prognostic biomarkers for AML patients before chemotherapy. However, expression of signature genes for stemness is not significantly changed in resistant cells, suggesting resistant cells may represent a subpopulation of cells in transition, rather than LSCs. In addition, we discovered that epigenetic inhibitors, such as histone deacetylase inhibitor (HDACi), Romidepsin, can effectively induce cell death of chemoresistant cells through targeting slow-growing CD47- and CD123-positive cell pools. Therefore, epigenetic inhibitors can not only be used to treat chemoresistant cells as standard chemotherapy agents, but can also be used to prevent the development of chemoresistance in AML cells.

Materials and methods

Cell lines

OCI-AML2, OCI-AML3, KG-1, MV4-11, THP-1, Molm-13, HL60, and HL60 Doxorubicin (Dox)-resistant cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen and American Type Culture Collection repositories and cultured under their instructions. All cell lines were verified by short tandem repeat analysis and tested for mycoplasma contamination.

AML patient samples

The AML patient cells were obtained and approved via Institutional Review Board of the University of Florida in accordance with the Declaration of Helsinki and cultured as previously described [19, 20]. Briefly, cells were cultured in Stemspan SFEM II supplemented with 100 ng/ml stem cell factor, 100 ng/ml Flt3 ligand, 100 ng/ml thrombopoietin, 20 ng/ml G-CSF, 20 ng/ml interleukin-3.

Chemicals used for cell treatment study

The following list of reagents were purchased and used in this study: Cytarabine (Sigma 1162002), Doxorubicin (Sigma D1515), Romidepsin (Cayman 128517-07-7), carboxyfluorescein succinimidyl ester (CFSE; BD 565082), BMIi (Millipore 5.30154.0001), DZNep (Xcessbio M60141-2s), and Givinostat (Apexbt A4093). JQ1 was provided by Dr. J. Brandner from Dana-Farber Cancer Institute.

Generating of Ara-C-resistant cell lines

Resistant cell lines were generated using the method as described previously [21]. Briefly, OCI-AML2 or MV4-11 cells were treated with increasing concentration of Ara-C starting from 50 nM until it reached the resistance of the drug concentration as indicated.

Fluorescence-activated cell sorting (FACS) staining and single-cell sorting

The flow cytometric analyses were performed with Accuri C6 (BD Biosciences, San Jose), and cell sorting was performed with FACS Aria II (BD Biosciences). Single-cell sorting was performed directly into 96-well plates with 150 µl culture medium per well.

RNA-sequencing (RNA-Seq) analysis

RNA was extracted using RNA purification kit (Genesee Scientific, San Diego). RNA libraries were prepared using the TruSeq RNA sample prep kit (Illumina, San Diego) and sequenced using Illumina HiSeq 2000 Sequencer (Illumina).

Transcript-level quantification of RNA-Seq data was carried out by Salmon [22] using Ensemble release 75 as the reference. Transcript-level quantification was aggregated to gene expression level using tximport R package. Gene

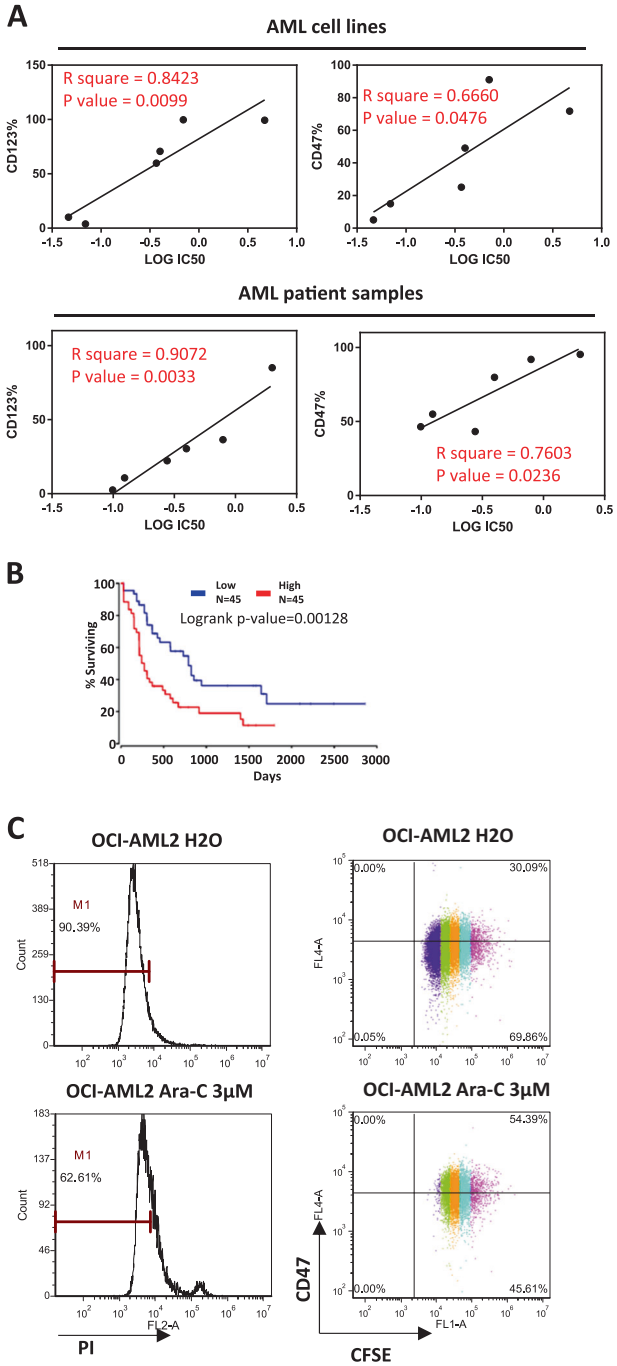


Fig. 1 Leukemia stem cell markers CD123 and CD47 are positively correlated with Ara-C sensitivity in AML cells and patient samples. **a** FACS analysis was performed with antibodies against stem cell surface markers CD47 and CD123 across cell lines (upper panel) and AML patient samples (see Table S1) (lower panel). Pearson's correlation coefficient of percentage of cell with LSC marker and Ara-C IC_{50} was calculated with GraphPad Prism 6. **b** Kaplan-Meier plot for CD123 (IL3RA) in AML patients. Patient data are from TCGA. $n = 45$ per group. **c** OCI-AML2 cells were first stained with cell tracing reagent carboxyfluorescein succinimidyl ester (CFSE) and cultured for another 3 days, and then treated with H_2O or 3 μM Ara-C for 24 h. Cells were stained with APC-CD47 and propidium iodide (PI). Live cells were gated by PI-negative population. Cells were gated based on CFSE intensity peaks. Stronger CFSE signal represents slower proliferating cells

length scaled TPM (transcripts per million) values were obtained by setting counts FromAbundance = "lengthScaledTPM" by tximport [23]. Gene set enrichment analysis (GSEA) was performed using Consensus PathDB-human database (http://www.geneontology.org/) [24, 25] and GSEA software [26].

Raw data and normalized gene expression data are deposited in the Gene Expression Omnibus database under accession numbers GSE108142 and GSE114649.

Cell proliferation assay

1×10^4 cells/well were seeded in Black 96-well Assay Microplate (Corning) with vehicle or drug containing culture medium, and further cultured for 72 h. The number of viable cells were tested with MTS assay kit (G5430, Promega), following the manufacturer's instructions. The absorbance was measured by Glomax®-multi+ detection system (Promega) at 490 nm. The cell viability was calculated by the comparison of the absorbance reading obtained from treated versus control cells.

Mouse xenograft studies

Xenograft studies were performed following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida (IACUC #201609309). OCI-AML2 and OCI-AML2 R50 cells were infected with lentiviruses produced from pMSCV-GFP-Luc plasmid. Then, 1×10^6 cells were injected into 8- to 10-week-old NSG mice (The Jackson Laboratory, 005557). Tumor burden was monitored weekly. For bioluminescence imaging, mice were given 150 mg/kg of D-luciferin-K (PerkinElmer 122799) in Dulbecco's phosphate-buffered saline (DPBS) by intraperitoneal injection. After 15 min of injection, bioluminescence was imaged with Xenogen In Vivo Imaging System (Caliper Life Sciences, Hopkinton, MA, USA).

For drug treatment, Romidepsin or Romidepsin and Ara-C combine treatment with indicated drug dosage was delivered through tail vein injection at day 0 and day 7 post implantation of tumor cells.

Statistical analysis

Student's *t*-test was used to analyze data from cell counting, cell viability, colony formation assay, and in vivo xenograft experiments. Values of $P < 0.05$ were considered statistically significant. Pearson's correlation coefficient of data sets was analyzed by GraphPad Prism 6.

Combined drug effect is quantified by combination index (CI) which was calculated by the Chou–Talalay algorithm with CompuSyn software (ComboSyn Inc.) [27]. A CI

value of 1 indicates an additive effect, CI <1 represents synergism, and CI >1 represents antagonism.

Results

LSC surface markers CD47 and CD123 are positively correlated to drug resistance

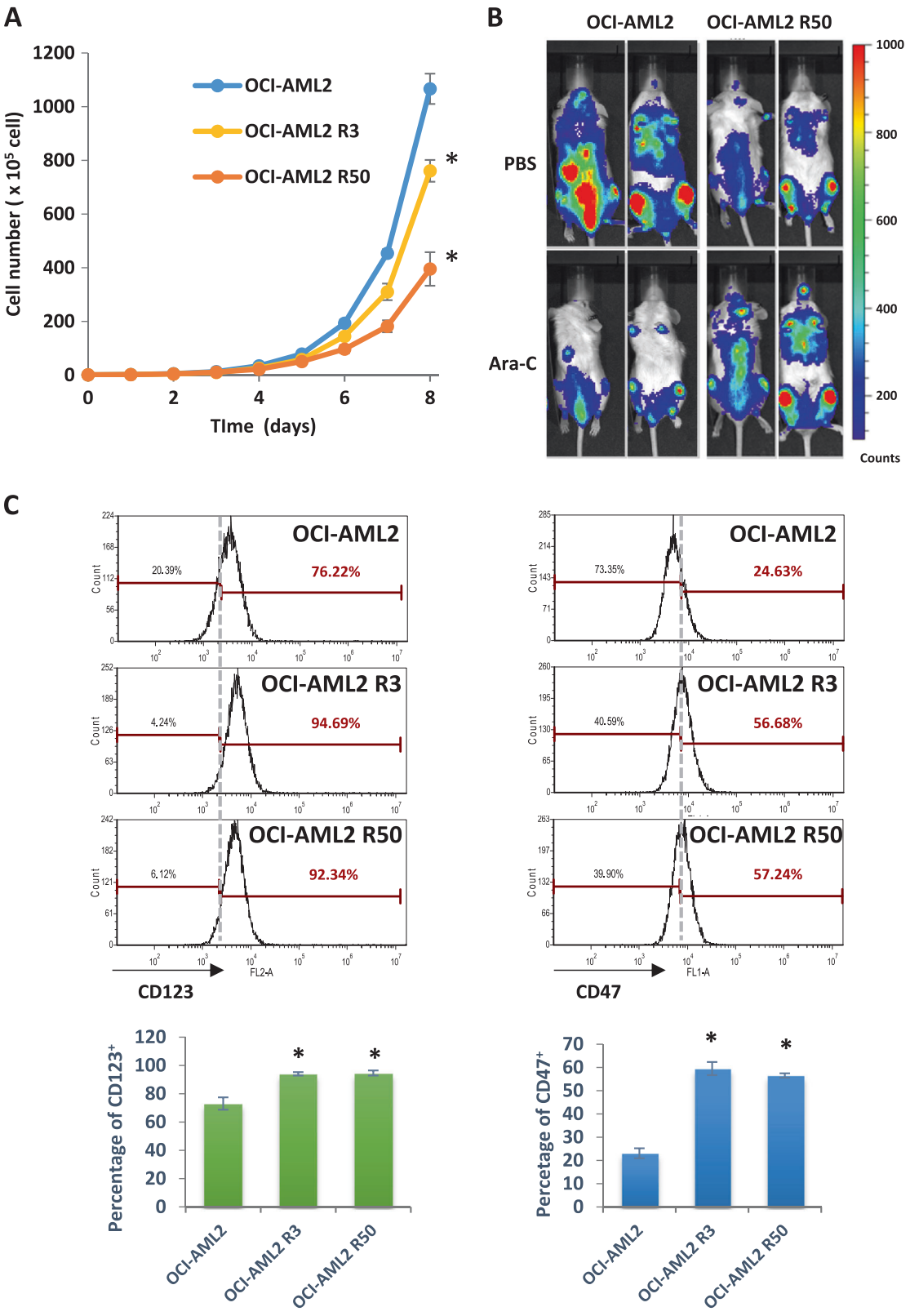
The recurrence after chemotherapy might lie in the intrinsic or acquired resistance of specific pool of AML cells, or alternatively in the existence of LSCs that are quiescent and not responsive to chemotherapy [2, 28]. Since LSCs are in low frequency and remain quiescent in patients, it is difficult to study the chemoresistant mechanism on this population. It has been previously shown that cell lines that derived from patient samples are considered as attractive models aimed to better characterize LSC biological properties and their chemotherapy resistance mechanisms [29]. Multiple LSC cell surface markers have been identified in patient samples and cell lines [6]. However, it remains to be determined whether LSC subpopulations with different LSC markers have similar level of chemoresistance and stem cell phenotype.

To better understand the mechanism of drug resistance and to determine whether chemoresistance is correlated to the expression of a certain group of LSC surface markers, we collected human AML cell lines and first examined correlations of their IC_{50} (drug dose causing 50% growth inhibition) to Ara-C and percentage of individual LSC surface markers in these cell lines (Fig. S 1A, S1B). We screened a panel of LSC markers that has been reported and well accepted [6, 30, 31]. To our surprise, the most frequently used LSC marker CD34 is not correlated with cell chemosensitivity. Other LSC surface markers, such as CD96, CD13, CD45RA, CD44, and CD117, are also not correlated to chemoresistance (Fig. S1C). Interestingly, the levels of CD47 and CD123 surface markers are positively correlated to chemoresistance (Fig. 1a, upper panel). These correlations are also confirmed in AML patient samples (Fig. 1a, lower panel, Fig. S1D and Table S1). Furthermore, patient survival data from The Cancer Genome Atlas (TCGA) also shows that a higher level of CD123 is strongly correlated with lower survival rate (Fig. 1b) [32]. We next tested the effect of Ara-C treatment on LSC population. OCI-AML2 cells were labeled with a stable cell tracking dye, CFSE, to track their proliferation rate. We found Ara-C treatment preferentially targets fast-growing, low CD47 population, resulting in the enrichment of slow-growing, higher CD47 marker population (Fig. 1c). FACS analysis data also showed Ara-C treatment preferentially target $CD47^{-}CD123^{-}$ cells and enriched $CD47^{+}CD123^{+}$ population (Fig. S1E). These results indicate that LSC surface markers CD47 and CD123 are positively correlated to the cell survival against chemotherapy drug treatment.

We next generated two drug-resistant cell lines from a drug-sensitive AML cell line, OCI-AML2, by gradually increasing Ara-C concentration in culture medium. The OCI-AML2-resistant lines were established to resist Ara-C in the concentration of 3 μM or 50 μM (OCI-AML2 R3 and OCI-AML2 R50) (Fig. S2A and Fig. 2a). These cells consistently grew slower in vitro compared to parental cells (Fig. 2a). The phenotypes of established resistant cells are stable even after drug withdrawal for a few months (Fig. S2B). Notably, the resistant cells derived leukemic blasts also grew slower and resisted to Ara-C treatment when engrafted into NSG mice (Fig. 2b). Consistent with the notion that $CD47^{+}$ and $CD123^{+}$ population are correlated with the level of drug tolerance, we found OCI-AML2 cells gradually increased CD123 marker during acquiring resistance (Fig. S2C). The percentage of CD123-positive cells increased to over 90% and CD47-positive cells also increased from 25% to over 50% in the resistant cells (Fig. 2c). Similarly, Ara-C-resistant cell line, MV4-11 R4, also have increased CD123- and CD47-positive population compared to parental cell line MV4-11 (Fig. S2D and Fig. S2E). In order to investigate whether the increase of $CD123^{+}$ and $CD47^{+}$ population also apply to other chemo drug-resistant AML lines, we tested HL60 R cells that are resistant to Doxorubicin (Fig. S2F). Consistently, the result shows that the resistant line HL60 R has a higher percentage of CD123 compared to parental cell line HL60 (Fig. S2G). These results demonstrate that AML chemoresistance is positively correlated to the percentage of LSC markers CD123- and CD47-positive cells.

Genes differentially expressed in drug-resistant cells are involved in cell cycle and DNA repair pathways

To gain further insight into the molecular mechanism underlying Ara-C resistance, we performed RNA-seq analysis to investigate the changes of gene expressions in resistant cells. Heatmap shows that OCI-AML2 R3 and R50 have a similar pattern of gene expression changes compared with the parental cell line OCI-AML2. For most of the changed genes, OCI-AML2 R50 shows higher fold alterations compared to that of OCI-AML2 R3 cells (Fig. 3a, left panel), indicating that altered genes are truly involved in drug resistance. Surprisingly, the expression of stem cell signature genes, such as OCT-4 and SOX2, are not significantly altered in resistant cells compared to sensitive cells (Fig. 3a, right panel and Fig. S3A). We also analyzed 17 LSC genes which were used to identify LSCs [33] and there are no significant changes in resistant cells compared to sensitive cells (Fig. S3B). Serial dilution study also showed that there is no significant difference between



sensitive line and resistant line in tumor initiating cell frequency (Table S2), indicating that although having higher population of cells expressing LSC markers, the resistant cells do not alter expressions of stem cell signature genes or acquire all the stemness phenotype. We then performed Gene Ontology (GO) analysis and pathway enrichment

◀ **Fig. 2** Ara-C-resistant cell lines grow slower, and maintain higher LSC markers. **a** Cell proliferation was tested between OCI-AML2, OCI-AML R3 (resistant to 3 μM of Ara-C), and OCI-AML R50 (resistant to 50 μM of Ara-C) cells for 8 days. Data are means ± SD ($n = 3$ independent experiments). *$P < 0.05$ compared to OCI-AML2 control. **b** Xenografts were visualized by luminescence after D-luciferin injection on day 14 with treatment of PBS (top) or 6 mg/kg Ara-C daily (bottom). One representative mouse from each group is shown ($n = 5$). **c** OCI-AML2 or resistant cells were stained with LSC marker CD123 or CD47 and analyzed by flow cytometry. An average number and SD from triplicated experiments are shown below. *$P <$ 0.05 compared to OCI-AML2 control

analysis on the genes differentially expressed in drug-resistant cells. These altered genes are enriched in cell cycle, DNA repair, and cell survival related pathways rather than stem cell related pathways (Fig. 3b and Fig. S3C). GSEA results also showed genes that differentially expressed in resistant cell lines are negatively correlated to intrinsic apoptosis signaling pathway and positively correlated with DNA repair (Fig. 3c). Therefore, the resistance mechanism may be mainly mediated through the alteration of cell cycle, DNA repair, and apoptosis pathways, instead of acquiring the stemness phenotype.

Romidepsin resensitizes resistant cells to chemotherapy in vitro

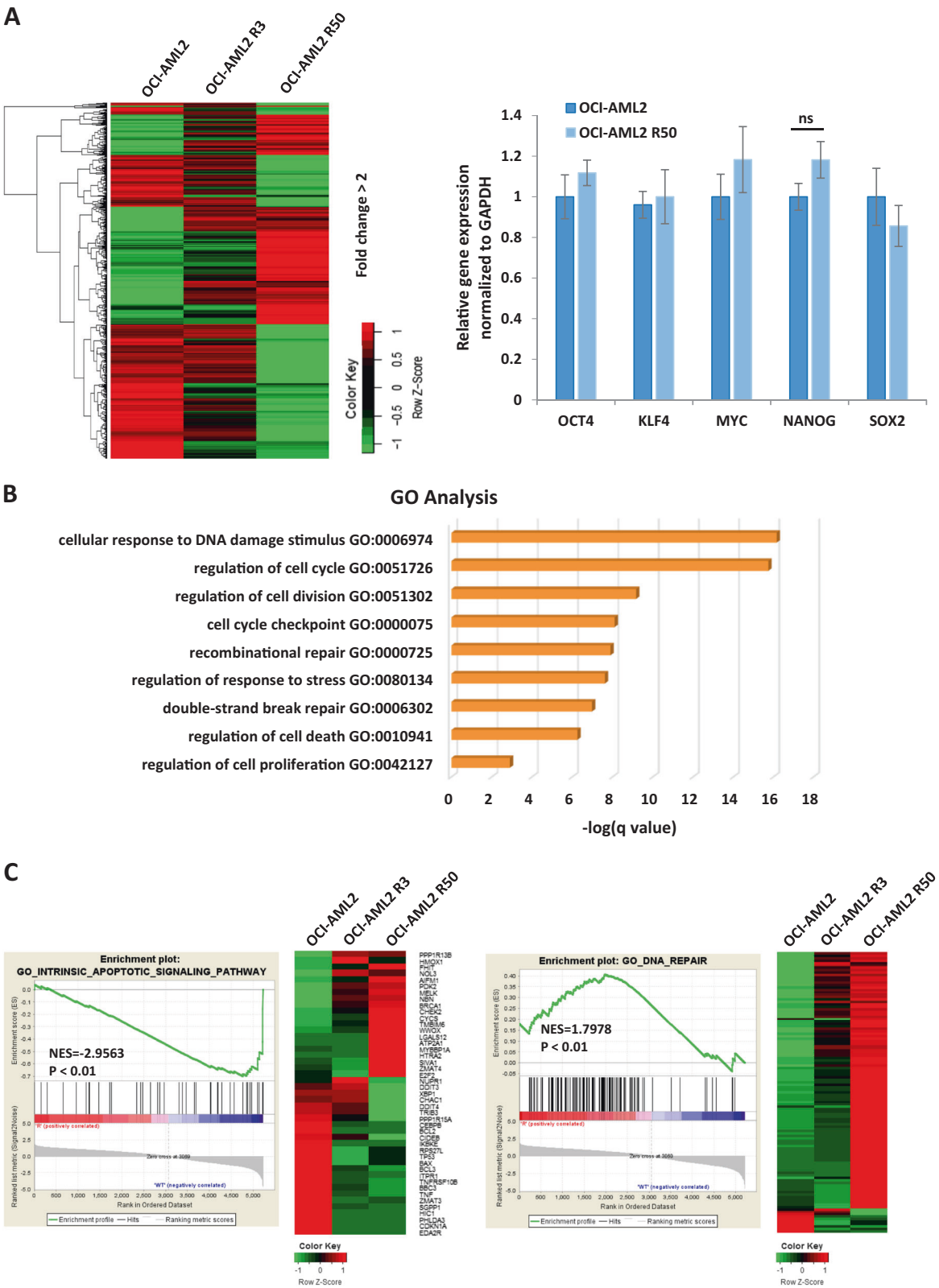
It has been shown that epigenetic drugs are effective for targeting drug resistance in solid tumor and other cancers, yet the detailed mechanisms remain elusive [34–36]. We therefore investigate whether selective epigenetic drugs are effective to induce cell death in OCI-AML2 R50 cells for a potential therapeutic approach to treat chemoresistant AML. The HDACis, Romidepsin and Givinostat, PRC1 complex inhibitor, BMIi, PRC2 complex inhibitor, DZNep, and bromodomain inhibitor, JQ1, were examined for their ability to kill the chemoresistant AML cells. The results show that all these drugs are effective for inducing cell death in both sensitive and resistant cells, although more efficient killing was seen in sensitive cells (Fig. 4a). We also observed synergistic effect when treated with Romidepsin and Ara-C together in OCI-AML2 sensitive cells (Fig. 4b). This synergistic effect was also seen in AML patient samples (Fig. 4c and Fig. S4A). Interestingly, Romidepsin can sensitize resistant cells to Ara-C treatment leading to a more significant synergetic effect in combination (Fig. 4d). This resensitization effect was also confirmed in another HDACi Givinostat (Fig. 4e). The increased cleaved poly (ADP-ribose) polymerase (PARP) further support that there are increased apoptosis in combined treatment (Fig. 4f). Similarly, the resensitization of drug resistance by HDAC inhibitor was also seen in the Doxorubicin-resistant HL60 cells (Fig. S4B). In contrast, other epigenetic drugs such as BMIi, DZNep, or JQ1 do not show effective resensitization in resistant cells (Fig. S4C). Taken together, Romidepsin has a combined effects when treated together with Ara-C in drug-sensitive strain and, more importantly, it can sensitize resistant cells to chemotherapy drug.

Romidepsin treatment largely reversed altered genes in chemoresistant cells

To gain further insight into the molecular mechanism underlying effect of HDACi treatment, we performed RNA-seq analysis to investigate changes of gene expressions in Romidepsin treated chemoresistant cells. Heatmap shows Romidepsin treatment increased the expressions of a large portion of genes that were suppressed in chemoresistant AML cells compared with parental cells. Similarly, it decreased a number of genes that were activated in the resistant cells (Fig. 5a). GO analysis shows genes down-regulated in the resistant cells are enriched in regulation of inflammatory response, leukocyte differentiation, regulation of apoptotic pathway, regulation of cell death, etc. These pathways are also enriched by Romidepsin increased genes (Fig. 5b). GSEA also shows that Romidepsin increased the expression of genes enriched in inflammatory response and apoptosis pathways (Fig. 5c, upper panels and Fig. S5A). Furthermore, the leading edge subset (most significant changed genes) shown in heatmap indicates that within each enriched pathway, majority of the most significantly increased genes by Romidepsin are repressed in the resistant cells. In addition, there is a significant overlap of enriched pathways between resistant cell increased genes and Romidepsin decreased genes, like DNA biosynthetic process, DNA repair, and so on (Figs. S5B, C). GSEA also shows Romidepsin decreased gene expression in these categories (Fig. 5c, lower panels and Fig. S5D). Furthermore, the leading edge subset in heatmap shows that within each enriched pathway, a majority of the most significantly decreased genes by Romidepsin are increased in the resistant cells. In summary, Romidepsin treatment can largely reverse altered genes in chemoresistant cells, leading to sensitization of resistant cell to Ara-C treatment.

Romidepsin and Ara-C target different cell subpopulations and result in a synergetic effect

Since HDACis effectively reversed gene changes and also induce cell death in the chemoresistant cells, which possess high LSC markers, we then further investigate whether HDACis can target LSC marker-positive cell population. Indeed, Romidepsin treatment significantly reduces CD123/CD47 LSC marker double positive population and results in an increase of double negative population in both sensitive and the resistant cells, while Ara-C treatment increases



double positive population (Fig. 6a, Figs. S1E, S6A). Other HDACis like Givinostat and SAHA were also tested, consistent with the finding in Romidepsin, and they also target CD123/CD47 LSC marker double positive population (Fig. S6B). Other epigenetic drugs are either not as effective in decreasing CD123/CD47-positive population, such as DZNep and JQ1, or do not target a specific population, such as BMIi (Fig. S6C). Consistent with the observation in the

◀ **Fig. 3** Drug-resistant cells have altered cell cycle and DNA repair pathways. **a** Heatmap (left panel) shows gene expressions of OCI-AML2 R3 and R50 cells compared to parental cell line. Real-time PCR (right panel) was performed to test stemness gene expressions in resistant cells compared to parental cell line. **b** GO (Gene Ontology) analysis was performed on differentially expressed genes in drug-resistant cells compared with OCI-AML2 parental cell line, using a two-fold expression change cut-off. The *P* values were corrected for multiple testing using the false discovery rate method and were presented as *q* value. Significantly enriched (*q* value < 0.05). **c** The gene expression data generated by RNA-seq were analyzed using Gene Set Enrichment Analysis (GSEA, http://www.broadinstitute.org/gsea/index.jsp). Two representative significantly enriched gene-sets are shown here. NES normalized enrichment score

resistant OCI-AML2 R50 cells, treatment of HL60 R cells with Romidepsin reduces the double positive population while Doxorubicin increases double positive markers as Ara-C did in OCI-AML2 cells (Fig. S6D). Similar results were also seen in Molm-13 and THP-1 cells (Fig. S6E). We further tested AML cells derived from AML patient samples. Consistent with results from cell lines, Ara-C treatment enriches LSC marker-positive cells, whereas Romidepsin treatment reduces this population (Fig. 6b, and Fig. S6F). In addition, unlike Ara-C treatment which targets fast proliferating cells (Fig. 1c), Romidepsin targets cells in all proliferation rates, and therefore the combination of Romidepsin and Ara-C not only eliminates fast-growing cells, but also targets slow-growing cells which survived from Ara-C treatment (Fig. 6c and Fig. S6G). Taken together, Romidepsin and Ara-C target different cell subpopulations and result in a synergetic effect.

Romidepsin can significantly prolong animal survival in chemoresistant xenograft AML mouse model, and combination treatment with Ara-C can further relieve the tumor burden

Given that Romidepsin and Romidepsin plus Ara-C can effectively target chemoresistant cells in vitro, we next examined whether these treatments are also effective in vivo. One million OCI-AML2 R50 cells were injected into NSG mice followed by vehicle control or drug treatment (Fig. 7a). Romidepsin treatment significantly reduces leukemia burden and combined treatment with Ara-C further clears the remainder of leukemia blasts (Fig. 7a, right panel). All PBS- or Ara-C-treated mice died before day 35, while mice from Romidepsin or combined treatment groups had significantly prolonged survival (Fig. 7b). Although mice from the combined treatment group weighted less and died earlier than the Romidepsin alone group possibly due to the toxicity effect, flow cytometric analysis of engraftments show that peripheral blood leukemic blast reduced significantly in Romidepsin group and further reduced in combined treatment (Fig. 7c), and similar results were also seen in spleen and bone marrow (Fig. S7A–D). In contrast, treatment with Ara-C alone in mice with OCI-AML2 R50 engraftment had significantly increased tumor burden (Fig. 7a, right panel, Fig. 7c and Fig. S7A–D). Taken together, Romidepsin can significantly prolong animal survival in chemoresistant xenograft animal model, and combination treatment with Ara-C can further relieve the tumor burden. Therefore, Romidepsin or combined treatment of Romidepsin and Ara-C may serve as a therapeutic option for the treatment of chemoresistant AML patients.

Discussion

In this study, we investigated the relationship of stem cell marker expression and chemoresistance. We show that chemoresistance is positively correlated with cells that have high population of cells expressing CD123 and CD47 LSC markers. It remains questionable whether these resistant cells are LSCs. Although these cells grow slower and have higher LSC markers, the expression of stem cell signature genes are not significantly increased and tumor initiation cells were not significantly increased in resistant cells. It is conceivable that these cells are not LSCs, and instead they may represent a cell population in a transition stage [37]. Nevertheless, these LSC markers represent the chemoresistant phenotype and can be used as a chemoresistant biomarker for research and patient treatment.

Although chromatin alterations are favorable for drug intervention, the mechanisms of cancer-specific dependencies on epigenetic regulators are not completely comprehended. It has been shown that histone deacetylase inhibitors, PRC2 complex inhibitors, and bromodomain inhibitors are effective in targeting cancer stem cells in various cancers [38–41]. In this study, we examined a panel of epigenetic drugs for chemoresistant therapy for AML. Our study shows that these drugs are also effective in treating chemoresistant AML cells. Especially, HDACi can sensitize resistant cells to Ara-C treatment by further inducing cell apoptosis. In fact, HDACi and Ara-C target different pools of cells. While Ara-C targets fast-growing, CD123-negative cells, HDACi targets CD123-positive cells, and therefore the combination synergizes the effect. The in vivo xenograft models also show effectiveness of HDACi treatment on the chemoresistant leukemia. However, although the combined treatment of Ara-C and HDACi Romidepsin further decreased tumor burden in animals, it does not show an advantage for the overall survival rate because of the cytotoxicity of Ara-C. It warrants further studies to optimize the combined treatment. We further investigate the mechanism of HDACi effect on resistant cells. Gene expression profile study shows that HDACi can largely reverse altered genes in resistant cells to sensitive state. The changes in gene expression are mainly in the

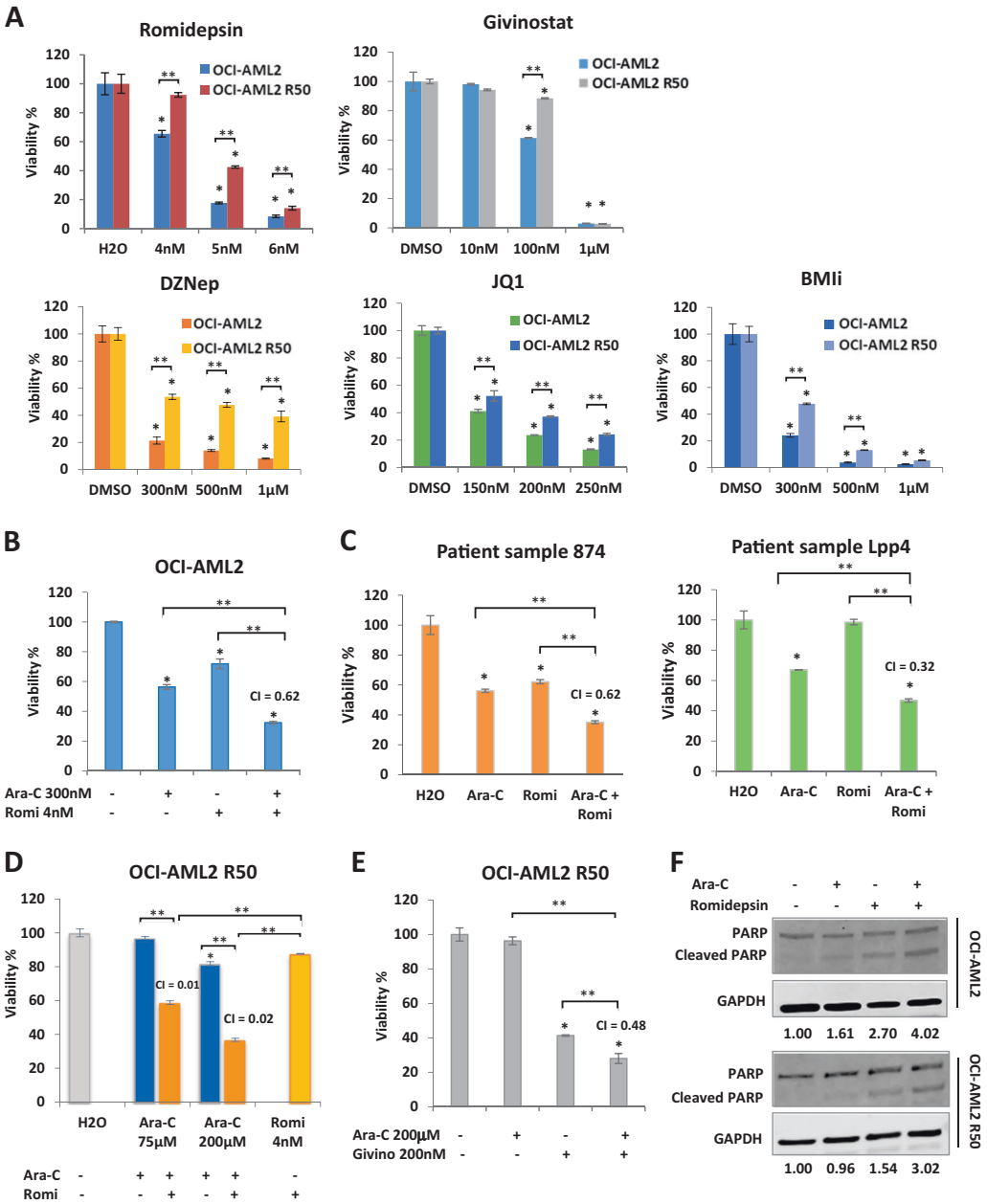
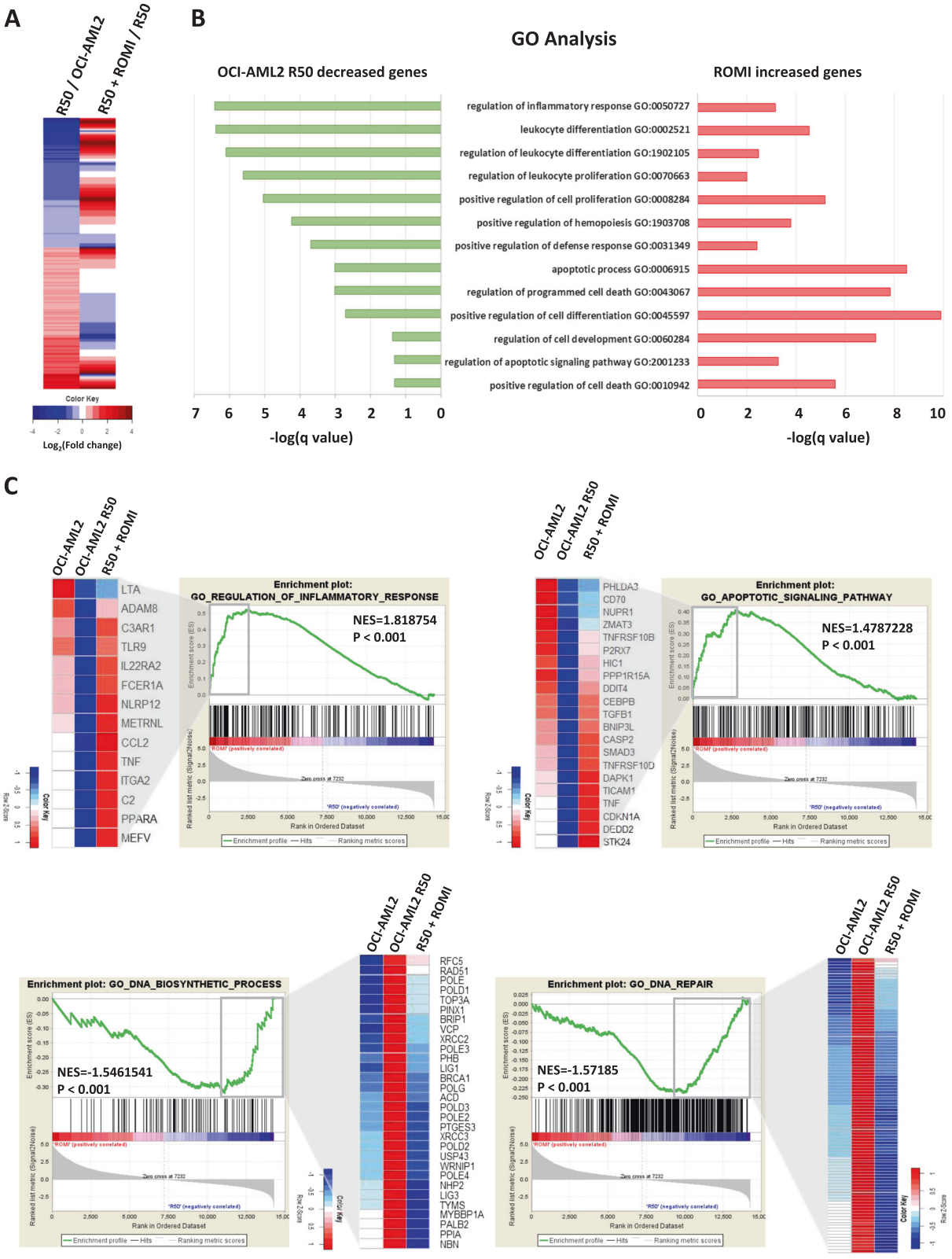


Fig. 4 Romidepsin resensitizes resistant cells to chemotherapy in vitro. **a** MTS assay was performed in OCI-AML2 and R50 cells treated with epigenetic inhibitors for 72 h. **b** MTS assay was performed in OCI-AML2 cells treated with Ara-C, Romidepsin, or combination. The data represent mean ± SD. $*P < 0.05$ (Student's t-test, treated vs control treatment), $**P < 0.05$ (Student's *t*-test between groups as indicated). Combination index (CI) value for combination of Ara-C and Romidepsin in OCI-AML2 cells were calculated with the Chou–Talalay algorithm by CompuSyn software. CI <1 indicates synergism. **c** Cell viabilities were tested by FACS with PI staining in AML patient samples after 24 h of treatment. CI <1 indicates synergism. **d** OCI-AML2 R50 cells were treated with HDAC inhibitor Romidepsin and Ara-C, MTS assay was performed after 72 h of treatment. CI <1 indicates synergism. **e** OCI-AML2 R50 cells were treated with HDAC inhibitor Givinostat combined with Ara-C and MTS assay was performed after 72 h of treatment. CI <1 indicates synergism. **f** Western blot detected PARP and cleaved PARP in OCI-AML2 and resistant cells with treatment as indicated for 24 h. The concentrations used in the treatment are 500 nM Ara-C, 2 nM Romidepsin in OCI-AML2 group, and 75 μM Ara-C, 4 nM Romidepsin in OCI-AML2 R50 group. The density signal was quantified by ImageJ. The numbers indicate the quantifications of cleaved PARP for each sample normalized by GAPDH loading control accordingly

category that regulates cell survival, DNA repair, and cell death regulation. Therefore, HDACi targets chemoresistant cells through the mechanism that reverse the cell to the sensitive state.

It has been reported that Wnt [42], Myc [43, 44], and HOXA9 [45] pathways contribute to LSC maintenance and drug resistance. However in our study, genes that activated in resistant cells did not enrich to Wnt pathway (Fig. S8A),



but enriched to Myc- and HOXA9-regulated gene pathways (Fig. S8B). More interestingly, these activation can be reversed by Romidepsin treatment (Fig. S8C). These further support our conclusion from a different angel that resistant cells may be not LSCs, but they can be reversed and targeted by HDACi. It also raise questions of how these

◀ **Fig. 5** Romidepsin treatment can reverse altered genes and pathways in drug-resistant cells. **a** Gene expression profiles were extract from RNA-seq data of OCI-AML2, OCI-AML2 R50, and OCI-AML2 R50 treated with Romidepsin for 18 h. Heatmap showing $\log_2$ fold change of gene expressions in resistant cells compared with its parental cells and changes of these genes upon Romidepsin treatment. **b** GO analysis on genes downregulated >1.5-fold in OCI-AML2 R50 cells (displaying in green) compared with OCI-AML2 parental cells and also genes upregulated >1.5-fold in OCI-AML2 R50 cells after treated with Romidepsin (displaying in red) compared with OCI-AML2 R50 cells. The *P* values were corrected for multiple testing using the false discovery rate method and were presented as *q* value. Significantly enriched (*q* value < 0.05). **c** GSEA of RNA-seq data from OCI-AML2 R50 and OCI-AML2 R50 treated with Romidepsin for 18 h. The leading edge genes altered by Romidepsin are shown in heatmap in comparison with OCI-AML2 and OCI-AML2 R50. Representative significantly enriched gene-sets are shown here. NES normalized enrichment score

pathways have been altered and it needs further studies to answer these questions.

It has been shown that patients with high percentage of CD123-positive blast at diagnosis had a lower percent of complete remission and higher percent of relapse [28, 46]. In fact, CD123 is present in almost all AML blasts and may be used as a unique single phenotype for minimum residual disease detection [47, 48]. It is reported that knockdown of CD47 also exerted a chemo sensitization effect in leukemia and solid tumors [49, 50]. Therefore, CD123 and CD47 stem cell markers can be served as biomarkers to predict responsiveness of chemotherapy and can also serve as a chemoresistant forecaster for relapse patients. Since CD123 is commonly present in minimum residual disease

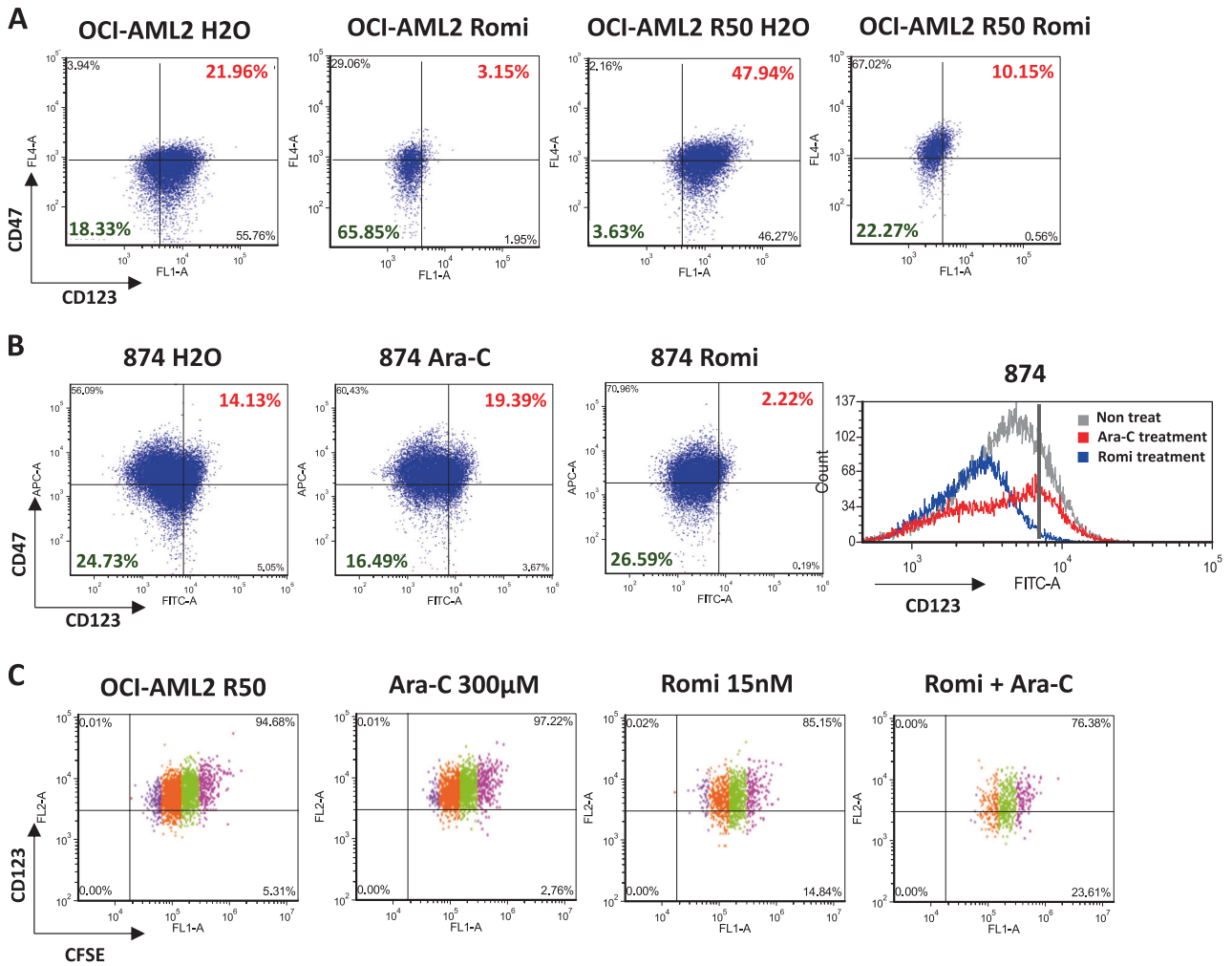


Fig. 6 Romidepsin and Ara-C target different cell populations. **a** FACS analysis of OCI-AML2 or OCI-AML2 R50 cells treated with Romidepsin or H_2O for 48 h. Live cells were gated by PI-negative population. **b** AML patient sample 874 was treated with H_2O, Ara-C or Romidepsin for 24 h. FACS analysis was performed with cells gated with PI-negative population. The change of $CD123^+$ population with drug treatment is shown in the right panel. **c** OCI-AML2 R50 cells were stained with CFSE, and cultured for 3 days, and then treated as indicated. Live cells were gated as PI-negative population and analyzed with CD123 and CFSE intensity peaks

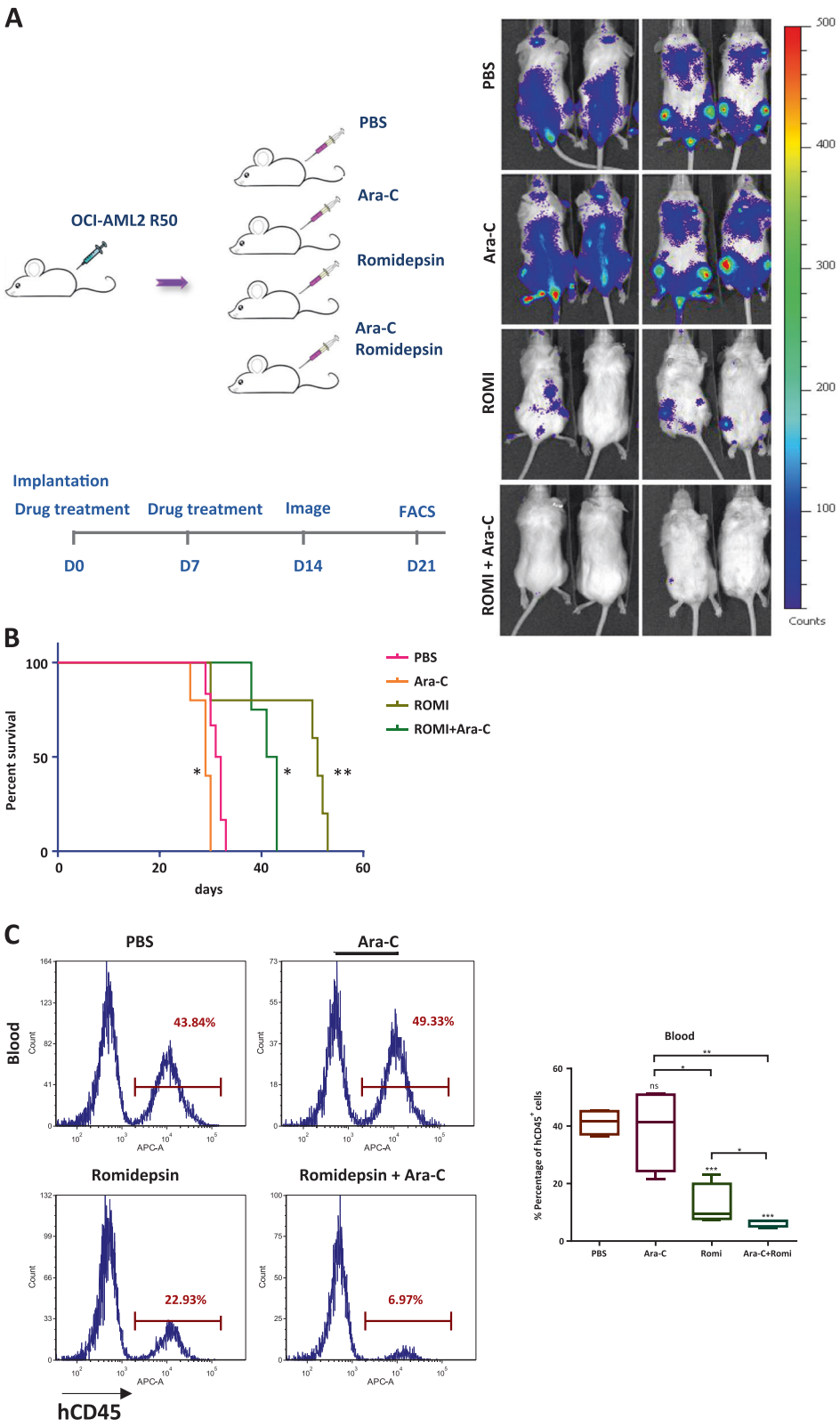


Fig. 7 Romidepsin can significantly prolong animal survival in chemoresistant xenograft animal model. **a** Schematic (left panel) overview of xenograft and drug treatment flow. Xenografts (right panel) were visualized by luminescence after D-luciferin injection (150 mg/kg) on day 14 with treatment of PBS, 3 mg/kg Ara-C, 1 mg/kg Romidepsin, or 3 mg/kg Ara-C combined with 1 mg/kg Romidepsin. Three representative mice from each group are shown ($n = 5$). **b** Kaplan–Meier survival curves of xenograft mice ($n = 5$ per group). **c** Representative figures of FACS analysis for peripheral blood from day 21 xenografts. Quantitative measurement of the AML tumor burden (hCD45+ cells relative to the total cells) in blood was shown in the right panel. The data represent mean ± SD; ns not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test between treatment groups and PBS group, except otherwise indicated)

and is linked to chemoresistance [31, 47, 48], targeting $CD123^{+}$ population may also be beneficial in preventing the development of chemoresistance in AML cells. Our data show that HDACi can target $CD123^{+}CD47^{+}$ cells, and also reverse gene changes in the resistant cells, which maintain a high LSC marker as a phenotype. Future experiments will

determine whether a low dose of HDACi will prevent or slow down the buildup of chemoresistance in AML cells. In summary, we demonstrate that CD123 and CD47 cell surface markers may potentially serve as biomarkers for chemoresistant AML cells. Our study also sheds light on a new mechanism of drug resistance in leukemia, and provides a rationale to develop and test epigenetic-targeted therapies in leukemia, especially in drug-resistant relapse patients.

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Author contributions BY, QC and KS performed experiments and contributed to experimental design. BY, MT, HL and AG contributed to the data analysis. JDK characterized and provided leukemic patient samples. BX, SH and YQ planned the research strategy and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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