Reversible switching of leukemic cells to a drug-resistant, stem-like subset via IL-4-mediated cross-talk with mesenchymal stroma

Ferrata Storti Foundation

Hae-Ri Lee,[1] Ga-Young Lee,[1] Eung-Won Kim,[1] Hee-Je Kim,[2] Minho Lee,[3] R. Keith Humphries[4,5] and Il-Hoan Oh[1]

[1]Catholic High-Performance Cell Therapy Center & Department of Medical Life Science, College of Medicine, The Catholic University, Seoul, Republic of Korea; [2]Division of Hematology, Department of Internal Medicine, St Mary's Hematology Hospital, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea; [3]Department of Life Science, Dongguk University-Seoul, Goyang-si, Gyeonggi-do, Republic of Korea; [4]Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada and [5]Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

ABSTRACT

Chemoresistance of leukemic cells has largely been attributed to clonal evolution secondary to accumulating mutations. Here, we show that a subset of leukemic blasts in contact with the mesenchymal stroma undergo cellular conversion into a distinct cell type that exhibits a stem cell-like phenotype and chemoresistance. These stroma-induced changes occur in a reversible and stochastic manner driven by cross-talk, whereby stromal contact induces interleukin-4 in leukemic cells that in turn targets the mesenchymal stroma to facilitate the development of new subset. This mechanism was dependent on interleukin-4-mediated upregulation of vascular cell adhesion molecule-1 in mesenchymal stroma, causing tight adherence of leukemic cells to mesenchymal progenitors for generation of new subsets. Together, our study reveals another class of chemoresistance in leukemic blasts via functional evolution through stromal cross-talk, and demonstrates dynamic switching of leukemic cell fates that could cause a non-homologous response to chemotherapy in concert with the patient-specific microenvironment.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous, clonal hematopoietic disorder characterized by excessive proliferation of stem cell-like progenitor cells in the bone marrow (BM). AML has a highly variable prognosis[1] and a very high risk of relapse particularly in elderly patients.[2]

Leukemia progression and relapse are widely viewed to occur via clonal evolution from preleukemic cells to overt leukemia driven by genetic mutations,[3] followed by additional mutations leading to treatment-resistant, relapsed clone(s).[4] However, in-depth clonal analyses have revealed the persistence of founding clones,[4] and functional heterogeneity among the developed leukemic clones,[5] suggesting that other mechanisms may be involved.

Several studies have highlighted leukemic stem cell (LSC) properties contributing to drug resistance:[6] AML patients whose leukemic blast exhibit higher levels of stem cell signatures are at greater risk of relapse and have a poorer prognosis.[7,8] However, the specific relationship between stemness and functional heterogeneity of LSC related to drug resistance, remains poorly understood.[7,9]

There is increasing awareness that the microenvironment, including growth factors, cytokines and niche stromal cells, can provide protection to leukemic cells and thereby contribute to the acquisition of chemoresistance.[10,11] For example, leukemic cell subsets surviving chemotherapy were localized to the surface of osteoblasts in the BM.[12-15] Subsequently, multiple protective signals from the stroma have been shown to enhance leukemic cell survival through activation of receptor tyrosine

kinases[16] or interaction with the extracellular matrix. However, despite these protective signals, a role for the stroma in the clonal development of leukemic blasts for the acquisition of chemoresistance has not been demonstrated.

Here, we show that subsets of leukemic cells in stromal contact undergo reversible changes associated with a stem cell-like phenotype and drug-resistant state. These changes are stochastic, and distinct from changes induced by other mechanisms of chemoresistance, thus representing a new class of drug-resistant cells developed in the leukemic microenvironment.

Methods

Human sample collection

Primary leukemic blasts were collected from newly diagnosed AML patients without prior treatment history. Part of the BM samples are from AML patients who had complete medical records during 5 years of follow-up in clinical courses. Human mesenchymal stromal cells (MSC) were separated from the BM of normal donors under informed consent. This study was approved by the Institutional Review Boards of St. Mary's Hospital and Catholic University of Korea.

Animals

C57/BL6 mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Bis$^{+/+}$, Bis$^{+/-}$, Bis$^{-/-}$ mice[17] were provided by Dr. Jeong-Hwa Lee (Catholic University of Korea). Mice with disruption of interleukin-4 (IL-4) receptor[18] were provided by Dr. Chang Yul Kang (Seoul National University).

Mouse and human acute myeloid leukemia cells and mesenchymal stromal cells

Fresh murine or human MSC were analyzed in the BM using flowcytometry. Cultured MSC (passage five to eight) were obtained by serial plating of BM cells in the DMEM containing 10% fetal bovine serum as described.[19,20] For generation of murine AML cells, fluorouracil (5-FU) treated BM cells were transduced with MN-1 or Meis1/HoxA9 through retroviral infection as described.[21,22] For co-culture, MSC were irradiated (15 Gy) 18-24 hours prior to use and leukemic cells were seeded on the MSC for co-culture. For co-culture with transwell, MSC were seeded into the upper chamber (6-well type, polyethylene terephthalate [PET] membrane with 0.4 µm pores; BD Bioscience, San Diego, USA) and leukemic cells were seeded into the lower well.

Flow cytometry of leukemic cells and mesenchymal stromal cells

Murine leukemic cells were analyzed by flow cytometry using the following antibodies: CD45.1-APC (BD PharMingen, USA), Lineage cocktail (StemCell Technologies Inc, Canada), Sca-1-PE-Cy7 and c-kit-PE (BD PharMingen). For human leukemic cells, CD45-APC, CD34-BV421, CD90-FITC (BD PharMingen) antibodies were used. For MSC, anti-CD106 (VCAM-1)-biotin, CD51-PE (eBioscience, CA, USA.), CD140a (PDGFRa)-APC, Sca-1-PE-Cy7 (BD PharMingen) were used.

Treatment of antibody and cytotoxic drug

Leukemic cells seeded on irradiated MSC were treated with anti-IL-4 antibody (R&D Systems Inc., USA), anti-CD106 (VCAM-1) (R&D Systems Inc.) for 3 days. For *in vivo* antibody injections, mice received intraperitoneal injection of anti-IL-4 Ab (1 mg/kg) (R&D Systems Inc.) or intravenous injection of anti-VCAM-1 antbody (10 mg/kg) (Bio X cell, USA) along with

immunoglobulin G (IgG) from rat serum (Bio X cell). Cytotoxicity of *in vivo* leukemic cells was examined by treatment with 100 mg/kg of Ara-C (Sigma-Aldrich, MO, USA) and 3 mg/kg of doxorubicin hydrochloride (Sigma).

Gene expression analysis

Sequencing libraries of two subjects were prepared according to the TruSeq Stranded Total RNA Sample Preparation guide. Aligned reads were quantified using HTSeq-count.[23] Differentially expressed genes, fold ratio, *P*-value, and false discovery rate were identified by edgeR algorithm[24] for each subject. Enriched KEGG pathways were identified by GSEA-P.[25]

Statistical analysis

In order to compare the generation of CD90+ subsets from individual primary human leukemia patients' samples, or the responses of individual patients' leukemia cells to chemotherapy, we used Mann-Whitney test. In order to compare the differences of means in specific experimental settings, we used a standard unpaired, two-tailed student *t*-test. The frequencies of leukemia-initiating cells in limiting dilution analysis were calculated by applying Poisson statistics with 95% Confidence Interval (CI) representing ±2 standard error of the mean (SEM).

Results

A subset of leukemic cells acquires a stem cell-like phenotype by contact with mesenchymal stroma

In order to investigate the influence of stromal cells on the function of leukemic cells, we employed an *in vitro* co-culture model of murine leukemic cells in contact with BM-derived MSC. Murine AML cells were generated by transducing BM mononuclear cells (MNC) with meningioma-1 (MN1)[21] or HoxA9-Meis1 (H9M1)[22] (Figure 1A). When co-cultured with MSC a subset of MN1 leukemic cells acquired a Sca-1(+) phenotype (Lin$^-$c-kit$^+$sca-1$^+$; LSK) mimicking normal hematopoietic progenitors, while the majority remained Sca-1(-) (Lin$^-$c-kit$^+$sca-1$^-$) (Figure 1B). The acquisition of Sca-1(+) phenotypes was similarly observed in other types of leukemic cells (H9M1) or leukemia cell line (C1498) independent of irradiation (*Online Supplementary Figure S1A and B*). The emergence of the Sca-1(+) subset was dependent on direct contact with the mesenchymal stroma (*Online Supplementary Figure S1C*), as these cells were not observed in stroma-free conditions or in stromal co-culture with a transwell filter (Figure 1B).

In order to determine if acquisition of the Sca-1(+) phenotype occurs *in vivo*, MN1 leukemogenic cells (Lin$^-$c-kit$^+$sca-1$^-$) were transplanted into mice. Consistent with the *in vitro* results, a subset of leukemic cells (GFP$^+$) in recipient mice acquired a Sca-1(+) (Lin$^-$c-kit$^+$sca-1$^+$) phenotype (Figure 1C).

In order to determine whether acquisition of the Sca-1(+) phenotype in leukemic cells originated from their fusion with stromal cells, as implicated previously,[26] we co-cultured MN1 leukemic cells (GFP$^+$) with MSC transduced with YFP. None of the GFP$^+$ leukemic cells co-expressed YFP (Figure 1D). Moreover, there was no difference in cell size between Sca-1(+) and Sca-1(-) cells, as determined by identical forward scatter in flow cytometry, and no increase in tetraploidy in the Sca-1(+) cells (Figure 1E). Similarly, there was no evidence of cell fusion in this *in vivo* generated Sca-1(+) subset (*Online Supplementary Figure S1D*).

A recent study implicated mitochondrial transfer from MSC to leukemic cells during acquisition of chemoresistance.[27,28] In order to examine this, MSC were labeled with a mitochondrial tracker and co-cultured with leukemic cells. There was no difference in mitochondrial tracker intensity between the Sca-1(-) and Sca-1(+) subsets (Figure 1F).

Altogether, this emergence of a new leukemic subset with a stem cell-like phenotype (Sca-1(+)) represents an intrinsic cellular evolution of leukemic cells that occurs independently of cell fusion or mitochondrial transfer during *in vivo* leukemogenesis and *in vitro* culture with stromal cells.

### Switching to the Sca-1(+) phenotype is reversible

In order to determine if the Sca-1(+) subset is a stable phenotype, we sort-purified Sca-1(+) (LSK) and Sca-1(-) (LK) leukemic cells generated during co-culture with stro-

ma, and replated for a second round of co-culture with or without stroma. The purified Sca-1(-) cell fraction again generated Sca-1(+) cells during the second round selectively in the presence of stroma, whereas purified Sca-1(+) cells co-cultured with stroma rapidly decreased in frequency (Figure 2A and B) with the emergence of a major Sca-1(-) cell population. Thus, final stable ratios of Sca-1(+) and Sca-1(-) cells were similarly maintained under secondary stromal co-culture conditions regardless of the phenotype of the initial cell population (Figure 2C). The changes in cell populations occurred rapidly within 3 days of co-culture suggesting that the conversion between Sca-1(-) and Sca-1(+) cells occurs by phenotypic switching rather than selective proliferation in the culture.

Thus, the emergence of Sca-1(+) leukemic cells during stromal contact occurs in a reversible manner in any subsets of leukemic cells without clonal predisposition (sto-
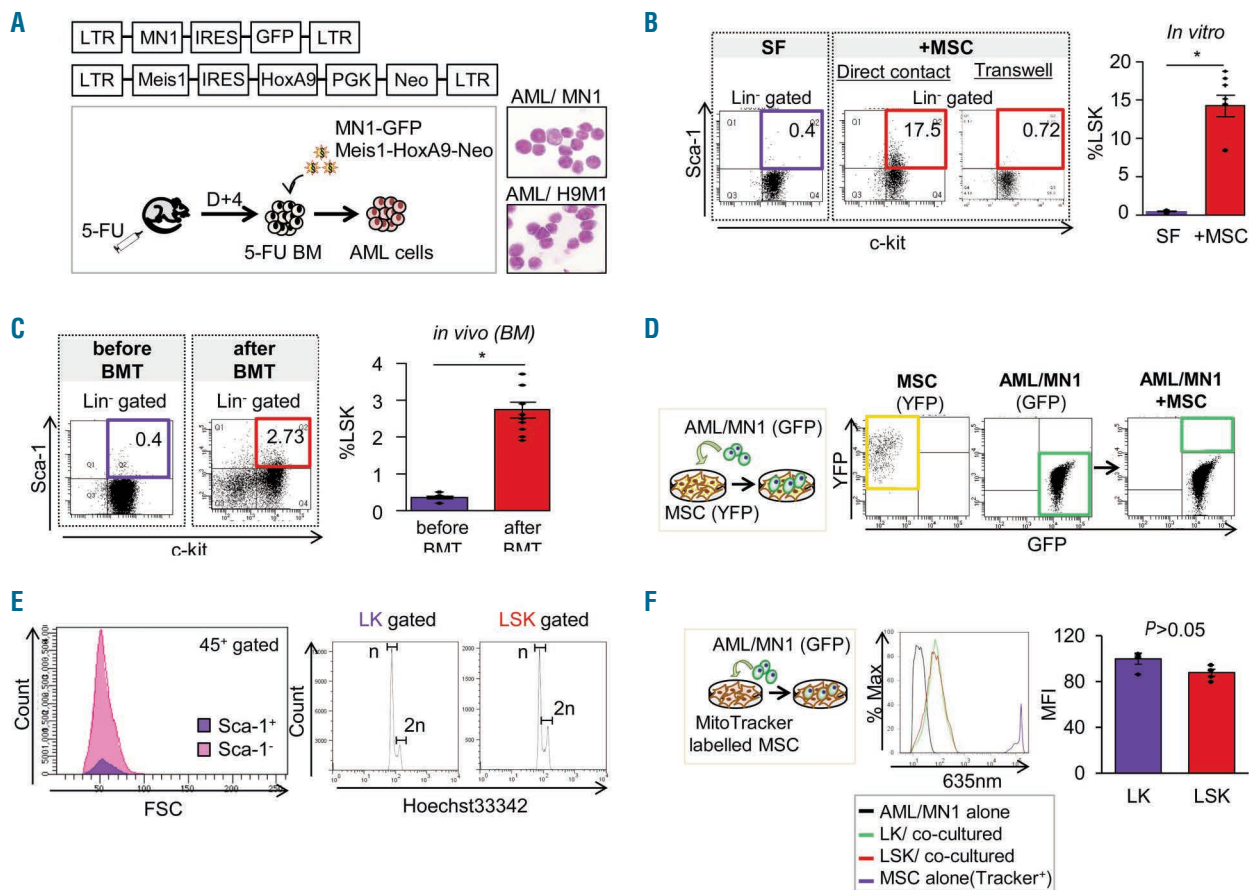
Figure 1. Generation of a stem cell-like phenotype in a subset of leukemic cells. (A) Schematic illustration of the experiment. Murine acute myeloid leukemia (AML) cells were generated by transduction of fluorouracil (5-FU)-treated bone marrow (BM) cells with retrovirus encoding oncogene (MN1, or HoxA9/Meis1). Shown are retroviral vectors, experimental procedure for transplantation into mice, and the light microscopy morphology of transformed leukemic cells visualized by Giemsa staining. (B) Generation of Sca-1(+) (Lin⁻c-kit⁺sca-1⁺: LSK) leukemic cells during co-culture with murine mesenchymal stromal cells (mMSC). Co-cultures with mMSC for 3 days were performed in the presence (transwell) or absence (direct contact) of a transwell membrane between the cells in comparison to stroma-free (SF) culture. Phenotypes of leukemic cells (CD45⁺GFP⁺) from co-cultured MSC (CD45⁻GFP⁻) were analyzed by flow cytometry. Shown are the representative profile (left) and quantification (right) (mean ± standard error of the mean [SEM] , n=7, *P<0.05). (C) *In vivo* generation of Sca-1(+) leukemic subsets. MN1 leukemic cells (Lin⁻c-kit⁺) were transplanted into mice and generation of Sca-1(+) subsets among BM engrafted leukemic cells were examined at 2 weeks post-transplantation (95% green fluorescent protein postive [GFP+] leukemic cells at the point). Representative flowcytometry plot (left) and quantification (right) are shown (mean ± SEM, n=10, *P<0.05). (D) Experimental scheme for analyzing cell fusion between MSC and leukemic cells. MSC transduced with a retroviral vector encoding yellow fluorescent protein (YFP), and leukemic cells transduced with a vector encoding GFP were co-cultured for 3 days. Shown are the experimental scheme (left) and representative flow cytometry profiles showing the absence of double positive (YFP/GFP) populations before and after co-culture (right). (E) Flow cytometry profiles for comparison between LK (Sca-1(-)), and LSK (Sca-1(+)) cell populations of cell size by forward scattering (left), and of DNA content (right) (n=5). (F) Experimental scheme to compare mitochondrial transfer between Sca-1(+) and Sca-1(-) cell populations. Murine MSC were pre-labeled with MitoTracker and co-cultured with MN1 leukemic cells for 3 days. Shown are representative flow cytometry plots from the experiments, each indicated leukemic cell subset (LK or LSK) of leukemic cells (CD45⁺GFP⁺) was gated and analyzed for MitoTracker and quantified for difference in mitochondrial transfer (n=4).
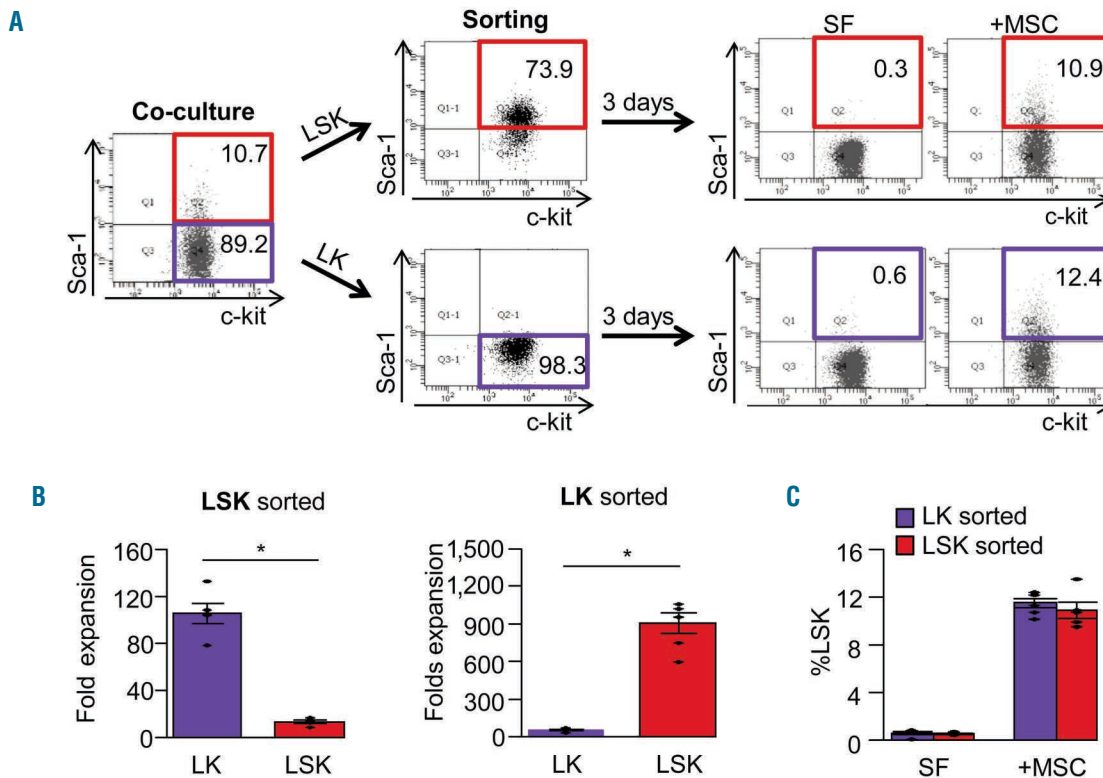
Figure 2. Reversible and equipotent nature for generation of LSK leukemic subsets. LSK (Sca-1(+)) or LK (Sca-1(-)) subsets of MN1 leukemic cells generated by co-culture were sort-purified and then replated for 3 days in the absence (SF) or presence of mesenchymal stromal cells (+MSC). (A) Flow cytometry profiles. (B and C) Quantitative analysis for expansion of cell numbers for LSK or LK subsets from input numbers of sorted LSK or LK cell populations during the second round of co-culture with murine MSC. Shown are the fold increases of cell numbers compared with input in the second co-culture (B) and final frequencies for LSK from each set of the second co-culture (C) (mean ± standard error of the mean, n=6, *P<0.05).

chastic), but with similar probability of each cells (equipotent) for conversion among the total leukemic cell populations.

Functional heterogeneity acquired in stem cell-like leukemic subsets

We next determined whether the Sca-1(+) stem cell-like leukemic subset arising by stromal contact was functionally distinct. When MN1 leukemic cells were treated with the chemotherapeutic Ara-C during co-culture with mesenchymal cells, Sca-1(-) subsets exhibited significant decrease of cell numbers, but the Sca-1(+) subset exhibited higher resistance compared to the Sca-1(-) subsets, with no significant changes in cell numbers (Figure 3A). Drug resistance in the Sca-1(+) subset was similarly reproduced in other leukemia cell types tested (HoxA9/Meis1-induced leukemic cells or C1498 leukemia cell line) (Figure 3A).

The chemoresistance of the Sca-1(+) (LSK) leukemic population compared to the rest of the Sca-1(-) (LK) cells was similarly observed *in vivo* with mice engrafted with MN1 leukemic cells and treated with chemotherapeutic drug (Ara-C and doxorubicin)[29] (Figure 3B). Thus, enhanced drug resistance is a common feature of leukemic subsets acquiring a Sca-1(+) phenotype upon stromal contact in a range of leukemic cell models.

In order to further investigate the drug resistance of the Sca-1(+) cells, we analyzed their cell cycling in BM and found that % of quiescent cell population (G0) was higher in LSK cells (Figure 3C). We also compared the frequency of leukemic initiating cells (LIC), a functional assay for

leukemia stem cells[30] in the Sca-1(+) leukemic subset in comparison to the other subsets. Thus, subsets of Lin(+) cells, Lin⁻c-kit⁻, LK (Lin⁻c-kit⁺sca-1⁻) cells and LSK (Lin⁻c-kit⁺sca-1⁺) leukemic cells generated in the BM of MN1 transplanted mice were sort purified and transplanted into secondary recipient mice in a limiting dilution assay. However, the LK and LSK populations exhibited a similar frequency of LIC, while exhibited significantly higher frequencies than the other cell populations (Figure 3D; *Online Supplementary Figure S2A*). These two populations (LK and LSK) also exhibited comparable levels of *in vivo* leukemic engraftment or *in vitro* leukemia colony formation (*Online Supplementary Figure S2B and C*), indicating that the Sca-1(+) subset developed during *in vivo* leukemogenesis comprise a subset of LIC that does not display significantly different leukemogenic activity compared to their Sca-1(-) counterparts.

Thus, the Sca-1(+) leukemia subset generated from leukemic cells represents a distinct leukemic cell population that has acquired drug-resistance without altering their leukemogenic activity.

Interleukin-4 plays a role in the emergence of drug-resistant Sca-1(+) cells

We next sought to identify possible signals from the stroma that induce emergence of Sca-1(+) cells. Given that altered production of cytokines and/or growth factors are frequently observed in leukemic cells,[10] we examined the cytokine/growth factor gene expression induced by stromal contact of leukemic cells (*Online Supplementary Figure S3A*).
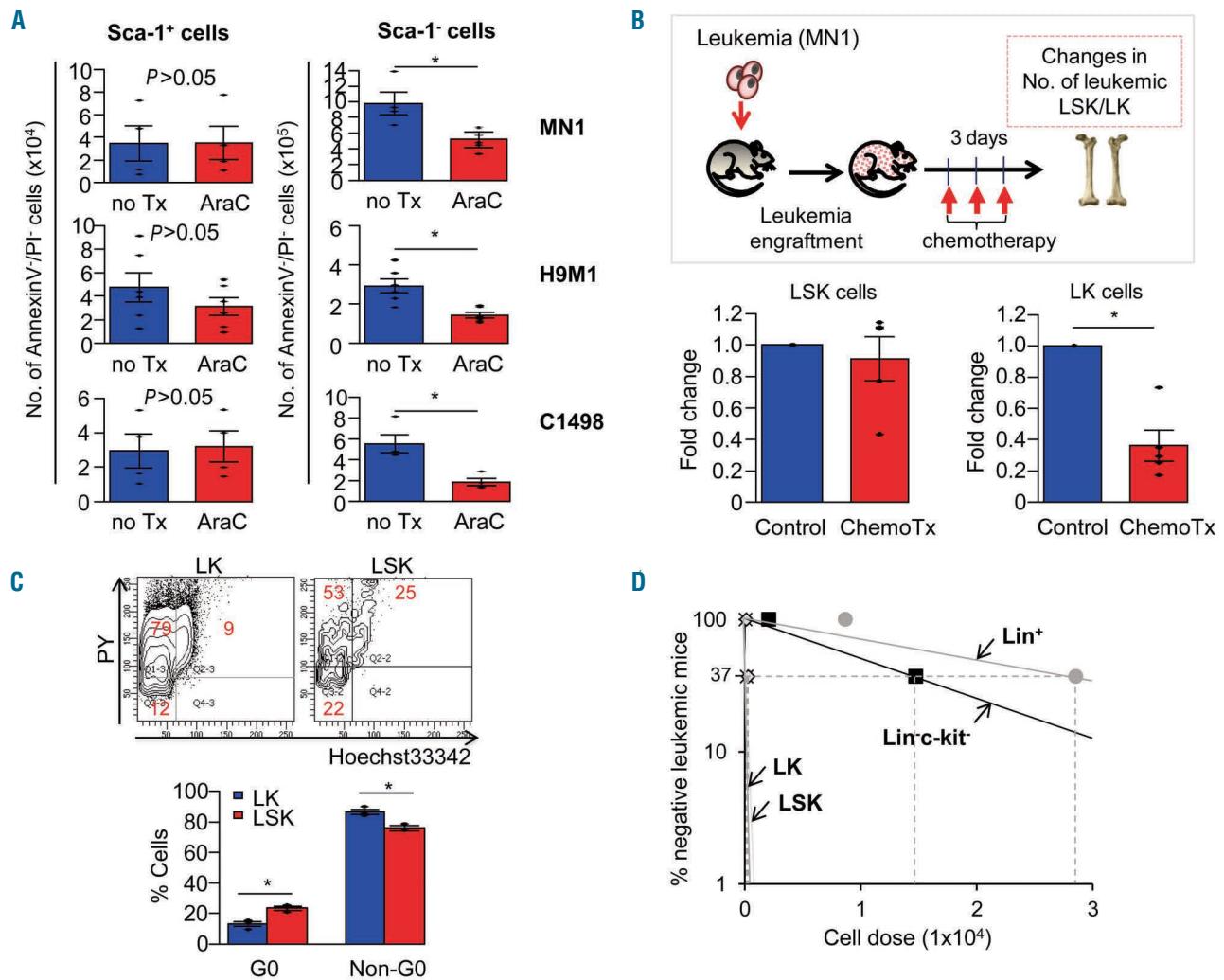
Figure 3. Property of stem cell-like leukemic subsets exhibiting drug resistance without changes in leukemic initiating cell frequencies. (A) Murine leukemic cells were cultured in the presence of murine mesenchymal stromal cells (MSC), and numbers of surviving cells (annexinV propidium iodide [PI]) in Sca-1(+) or Sca-1(-) subsets were measured after 2 days of treatment with Ara-C (100 nM for MN1 leukemia, 30 nM for H9M1, 500 nM for C1498 leukemic cells). (B) Mice engrafted with MN1 leukemic cells were treated with chemotherapeutic agents (Ara-C + doxorubicin) for 3 days by intraperitoneal injection at the indicated times and then examined for changes in the numbers of Sca-1(+) (LSK) or Sca-1(-) (LK) subsets in the bone marrow (BM). Experimental scheme (upper) and relative fold changes (lower) in the cell numbers after chemotherapy (chemoTx) compared to the control groups (mean ± standard error of the mean, n=5, *P<0.05). (C) Cell cycling of leukemic subsets in BM. MN1 leukemic cells were transplanted into mice and cell cycling of Sca-1(+) and Sca-1(-) subsets in BM were analyzed by Hoechst33342/pyronin staining. Shown are the representative flow cytometry plots with % of cell population (upper) and quantification of cells in G0 (quiescent cell population) and Non-G0 (G1/S/G2M) phase (lower) (n=3, *P<0.05). (D) Comparisons of leukemia-initiating cell (LIC) frequencies for each leukemic subset. MN1 leukemic cells were transplanted into mice and each subset of leukemic cells in recipient BM were sort-purified for transplantation into secondary recipients in a limiting dilution dose. Shown is the plot of limiting dilution analysis for frequencies of LIC in each leukemic subset analyzed by Poisson statistics. The resulting LIC frequencies are shown in the *Online Supplementary Figure S2A* with 95% Confidence Intervals in parenthesis.

Upon contact with stroma, the murine leukemic cells exhibited a notable induction of cytokines and growth factors implicated in leukemogenic activity, including IL-4, PDGF-A, PDGF-D, CCL-2, CCL-5, CXCL-1 and stem cell factor,[31-39] but not in the presence of transwell filters (*Online Supplementary Figure S3B*). Among those cytokines, IL-4 was selectively induced in LSK subsets, but not in the majority of remaining cells (LK) as determined by its transcript and protein level (*Online Supplementary Figure S3C and D*). Thus, we examined whether IL-4 acts as an autocrine signal for generating Sca-1(+) subsets. Addition of recombinant IL-4 increased the frequency of Sca-1(+) subsets (LSK) in a dose-dependent manner (Figure 4A). Conversely, addition of an IL-4-neutralizing antibody significantly decreased the frequency of LSK during co-culture (Figure 4A). Injection of an antibody against IL-4 into recipient mice along with MN1 leukemic cells also decreased the LSK population in the BM

of recipients without changes in overall engraftment levels (Figure 4B). Moreover, Il-4-neutralizing antibody abrogated resistance of the LSK population to chemotherapeutic drugs (Ara-C and doxorubicin), markedly decreasing the LSK population in recipient BM (Figure 4C), which caused a decrease in the residual burden of surviving LIC that can initiate leukemogenesis (*Online Supplementary Figure S4*).

Together, these results support a key role for IL-4 in the generation of drug resistant Sca-1(+) subset upon stromal contact.

Interleukin-4-dependent generation of Sca-1(+) leukemic cells is generated by stromal cross-talk

In order to investigate the mechanisms underlying IL-4-mediated generation of Sca-1(+) subsets, we examined the cellular target of IL-4 during the co-culture of leukemic cells and stroma (Figure 5A). First, to see if IL-4 acts directly on
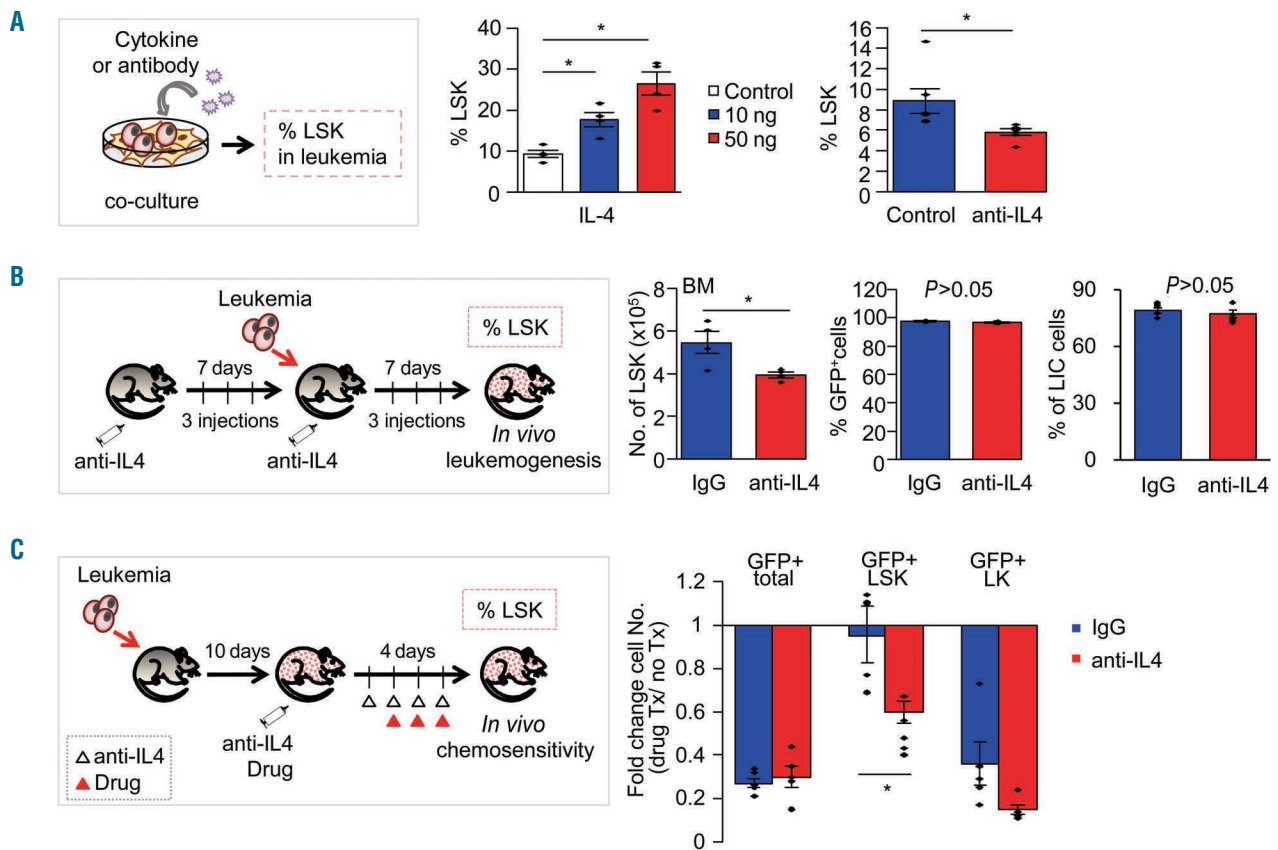
Figure 4. Role of stroma-induced interleukin-4 in the generation of the stem cell-like leukemic subset. (A) Effects of interleukin-4 (IL-4) during co-culture of leukemic cells. Left: experimental scheme; middle: % LSK generated during 3-day co-culture of leukemic cells with stroma supplemented with recombinant IL-4 (mean ± standard error of the mean [SEM], n= 6); Right: effects of antibody against IL-4 on generation of LSK during stromal co-culture of leukemic cells. Shown are the mean±SEM for % LSK in leukemic cells (green fluorescent protein positive [GFP+] CD45⁺) (n=7, *P<0.05). (B) Effects of IL-4 antibody on *in vivo* generation of LSK. Left: experimental design. Antibody against IL-4 was intraperitoneally administered into recipient mice at each indicated time point before and after transplantation of MN1 leukemic cells. Middle: numbers of LSK leukemic (GFP⁺) cells in the BM (two femurs and two tibia) of recipient mice. Middle and right: % of MN1 leukemic cell engraftment determined by total leukemic cells (GFP+ cells) (middle) and cells with leukemia-initiating cell properties (LK and LSK) (right), respectively (mean ± SEM, n=6, *P<0.05). (C) Effects of IL-4 antibody on the chemosensitivity of the leukemic subsets. Left: experimental design. After engraftment of MN1 leukemic cells (10 days after transplantation), recipients were injected with IL-4 antibody and chemotherapeutic drug (AraC+doxorubicin) at the indicated times. Right: changes in the chemosensitivity of leukemic subsets by *in vivo* injected IL-4 antibody. Relative fold decrease in the cell numbers of each leukemic subset compared to the control (phosphate buffered saline) group 3 days after exposure to drug and antibody (mean ± SEM, n=5).

leukemic cells, we established MN1 leukemic cells from hematopoietic progenitors of mice lacking IL-4 receptor α (IL-4Ra knockout [KO]). Co-culture of leukemic cells from IL-4Ra KO or wild-type (WT) with stromal cells led to comparable frequencies of LSK or LK subsets in each group (Figure 5B). In contrast, when mesenchymal stromal cells from IL-4Ra KO mice were co-cultured with MN1 leukemic cells, significantly lower frequencies of LSK, but not LK subsets, were observed compared to the WT stroma group (Figure 5C). Thus, IL-4 signals target mesenchymal stromal cells, rather than leukemic cells, to facilitate stroma-mediated generation of the LSK subset, indicating that IL-4-mediated cross-talk promotes the functional evolution of leukemic cells.

Next, to investigate the effects of IL-4 on mesenchymal stroma, we examined whether the mode of cellular interaction between MSC and leukemic cells is influenced by IL-4. LSK subsets were predominantly generated among the leukemic cells tightly adherent to the mesenchymal cells, for both MN1 or H9M1 leukemic cells, but seldom among the loosely adherent/suspension leukemic cells (*Online Supplementary Figure S5A*). Supporting the influence of IL-4 on stromal adherence, the level of vascular cell adhesion

molecules 1 (VCAM-1) in MSC, which mediate stromal adherence of leukemic cells,[40] were up-regulated by IL-4 in WT MSC, but not in IL-4Ra KO MSC (Figure 5D). Conversely, *in vivo* injection of IL-4-neutralizing antibody caused a significant decrease of VCAM-1 expressions in BM mesenchymal cells including subsets enriched for mesenchymal progenitors (CD44(-)PDGFRa(+))[41-43] (Figure 5E).

In order to further examine the influences of stromal VCAM-1 expression level on the generation of LSK subsets, leukemic cells were co-cultured with sort-purified MSC fractions for different levels of VCAM-1. MSC with higher VCAM-1 levels increased LSK generation during co-culture, whereas MSC expressing lower levels of VCAM-1 decreased it, in comparison to LSK cells from unsorted MSC co-cultures (Figure 5F). Similarly, VCAM-1-blocking antibody significantly decreased stromal adherence of leukemic cells (*Online Supplementary Figure S5B*), which led to a concomitant decrease in the generation of the LSK subset (Figure 5G). Moreover, *in vivo* administration of VCAM-1 antibody caused a significant decrease of LSK numbers in BM (Figure 5H). These data, together with positive expression of VCAM-1 ligands in leukemic cells (*Online Supplementary Figure S6*) indicates that tight adherence of
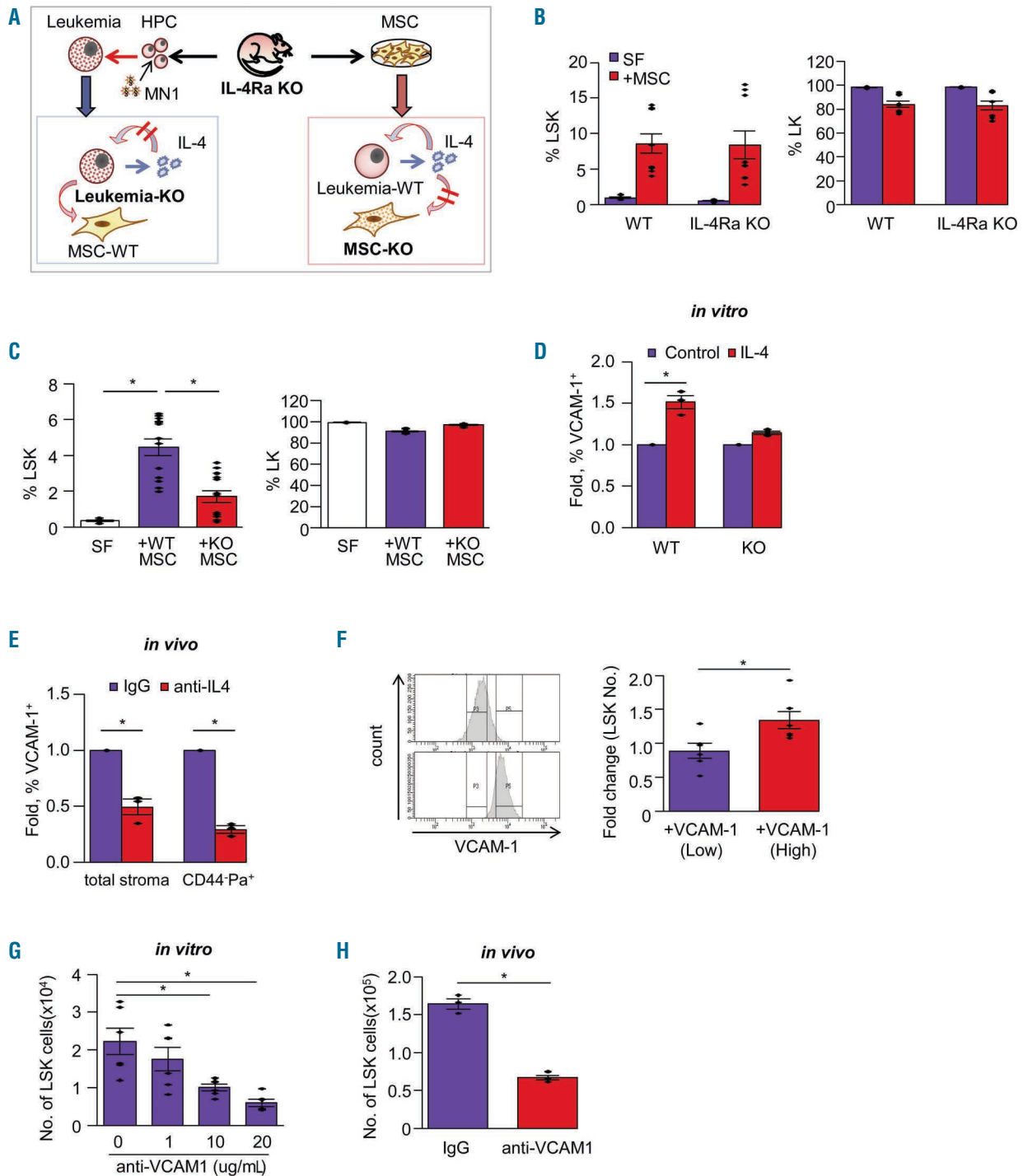
Figure 5. Interleukin-4-mediated cross-talk in mesenchymal stromal cells controlling VCAM-1-mediated generation of leukemic subsets. (A to C) Identification of interleukin-4 (IL-4) target in cross-talk of leukemic cells and mesenchymal stromal cells (MSC). (A) Experimental design. IL-4 receptor (IL-4Ra) knockout (Leukemia-KO) or wild-type (WT) leukemic cells were co-cultured with WT mice-derived murine MSC (MSC WT) (left), or MSC from IL-4Ra KO mice (MSC KO) or WT (right) were co-cultured with leukemic cells from WT mice. (B) Effects of IL-4Ra KO out in leukemic cells on the generation of LSK subsets. Shown are the quantification of % LSK and LK cells during co-culture (mean ± standard error of the mean[SEM], n=9, *P<0.05). (C) Effects of IL-4R KO in MSC on the generation of LSK subsets. Shown are the quantification of % LSK and % LK from the co-culture (mean± SEM, n=15, *P<0.05). (D to H) IL-4 targeting of MSC facilitates generation of leukemic subsets by controlling VCAM-1 expression in MSC. (D) Effects of IL-4 signals on VCAM-1 expression levels of MSC. Murine MSC from WT or IL-4R KO mice were treated with recombinant IL-4 and the fold increase of % VCAM-1(+) were analyzed in comparison to the control group (mean± SEM, n=6, *P<0.05). (E) In vivo changes of VCAM-1(+) cells in the bone marrow (BM) of mice injected with IL-4-neutralizing antibody. Mice were intraperitoneally injected with IL-4 antibody for 4 days and analyzed for % VCAM-1(+) cells in BM stromal cells. Shown are the relative fold differences of % VCAM-1(+) cells in the indicated subsets of BM mesenchymal stromal cells relative to the IgG treated mice group (n=5, *P<0.05). (F) Influence of VCAM-1 expression in MSC for generation of LSK subsets. Murine MSC were sort-purified for differences in VCAM-1 expression levels, and the generation of LSK subsets from MN1 leukemic cells co-cultured with each fraction was analyzed. Shown are the flow cytometry plots for sorting of MSC (left) and relative folds for LSK numbers generated in each co-culture group compared to co-culture with unsorted MSC (right) (n=6). (G) Effects of blocking antibody against VCAM-1 on the in vitro generation of LSK subsets. During co-culture of leukemic cells with stroma, the indicated amount of antibody against VCAM-1 was added and changes in the numbers of LSK generated in the co-culture were analyzed (n= 6, *P<0.05). (H) Effects of blocking antibody against VCAM-1 on the in vivo generation of LSK subsets. Mice transplanted with MN1 leukemic cells were injected with rat immunoglobulin (Ig) or VCAM-1-blocking antibody (intravenous 10 mg/kg) (7 and 10 days after leukemic cell transplantation). Three days after antibody injection, generation of LSK in recipient BM was analyzed (n=3 for IgG, n=4 for anti-VCAM1, *P<0.05).
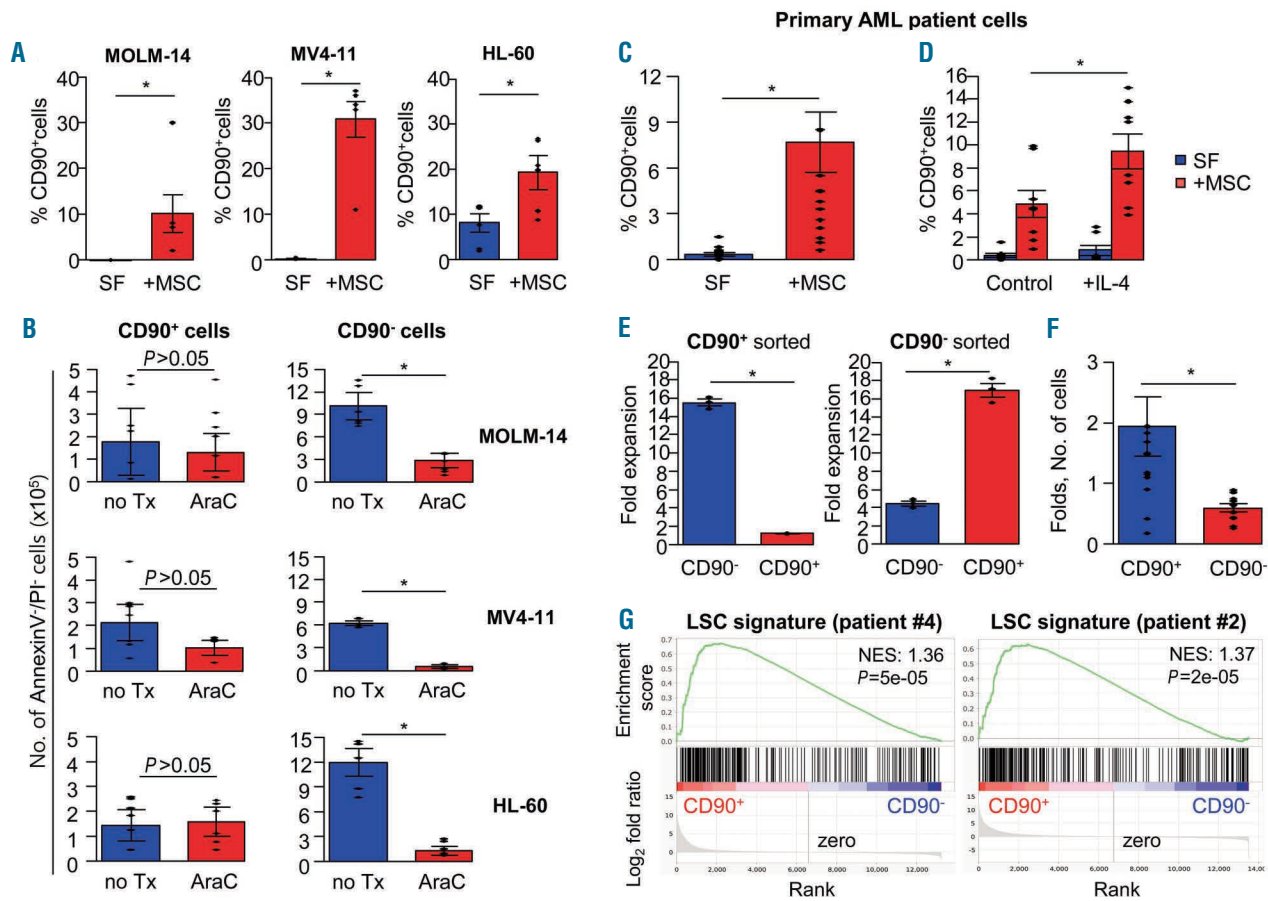
Figure 6. Generation of stem-like, drug-resistant leukemic cells in human leukemia models. (A) Generation of stem-like (CD90(+)) leukemic subsets from each indicated human leukemia cell line during stromal co-culture. Leukemic cell populations (CD45(+))were gate separated from mesenchymal stromal cells (MSC) (CD45(-) for analysis. Shown are the quantification of the frequency of CD90(+) cells in the co-culture (n=7, † <0.2%, *P<0.05). (B) Comparison of drug sensitivity between each leukemic subset. Each indicated human leukemic cell line was exposed to Ara-C (200 nM) for 2 days. Shown are the numbers of surviving (annexinV- propidium iodide-) cells in the culture (mean ± standard error of the mean [SEM], n=6). (C to G) Generation of stem-like, drug-resistant leukemic subsets in human leukemic cells from acute myeloid leukemia (AML) patients. (C) Human leukemia cells from AML patients were co-cultured in the presence or absence of human MSC. Shown are the % CD90 (+) cells in total leukemic cells after co-culture for 3 days and differences were analyzed by Mann-Whitney U test (n=14 from seven individual patients' samples, *P<0.05). (D) Effects of interleukin-4 (IL-4) on the generation of CD90(+) subsets. Leukemic cells were co-cultured with human MSC in the presence or absence of IL-4 (100 ng/mL). Shown are the relative fold increases in % of CD90(+) subsets in leukemic cells (mean± SEM, n= 10 from five individual patient's samples, *P<0.05). (E) Reversible switching of subsets of human primary leukemic cells to maintain constant equilibrium. CD90(+) and CD90(-) subsets generated during co-culture were sort-purified and re-plated in the co-culture with human MSC. Shown are the fold changes of each subset after plating each purified subset after 3 days of co-culture (n=6, *P<0.05). (F) Comparisons of drug sensitivity between the CD90(+) subset and the rest of the CD90(-) population in primary AML cells after exposure to Ara-C (200 nM). Shown are the relative fold changes in numbers of surviving cells of each population determined by numbers of annexinV-propidium iodide- (PI) cells and differences were analyzed by Mann-Whitney U test (n=14 from seven individual patients' samples, *P<0.05). (G) Enrichment of stem cell signatures in CD90(+) human leukemic cells. Primary human leukemic cells from two AML patients (#4 and #2) were co-cultured with human MSC for 3 days, and the generated CD90(+) and CD90(-) cells were subjected to RNA sequencing analysis. Differentially expressed genes were analyzed by gene set enrichment analysis (GSEA-P) for enrichment of 259 genes specific for leukemia stem cells.[47] Shown are the plots of enrichment scores (upper) and ranked list of each gene in the order of log. folds ratio (CD90+/90-) with position at zero indicated (lower).

leukemic cells to VCAM-1 in MSC facilitates emergence of LSK subsets.

Consistent with these findings, gene expression changes in MSC induced by IL-4 treatment during culture revealed 41 differentially expressed genes (DEG), the most profound changes of which were in the gene ontology group related to the 'binding' molecular function, supporting their role in the cellular interaction with leukemic cells (*Online Supplementary Figure S7A, B*). Thus, IL-4 enhances the cellular interaction of stroma and leukemic cells to facilitate stroma-dependent evolution of the Sca-1(+) leukemic subset exhibiting drug resistance.

Stroma-induced changes in human leukemic cell models

In order to investigate whether a similar phenomenon

can be seen in human leukemic cells, we examined human AML cells for acquisition of CD90(+) as a phenotype for stem-like subsets based on findings that a subset of CD90(+) cells amongst CD34(+) cells represent long-term repopulating hematopoietic stem cells (HSC)[44] and that CD90 expression in human leukemic cells represents high-risk leukemia with stem cell properties.[45,46] We first examined human leukemic cell lines, MOLM-14 and MV4-11 (M5 type FAB), and HL-60 (M3 type FAB). For each leukemic cell line tested, co-culture with human BM-derived MSC resulted in the emergence of leukemic subsets with the CD90(+) phenotype, albeit to variable levels (Figure 6A). Moreover, when chemoresistance was compared between leukemic subsets, significant resistance to Ara-C treatment was observed selectively for CD90(+) cells in all tested leukemic cells (Figure 6B) similarly exhibiting
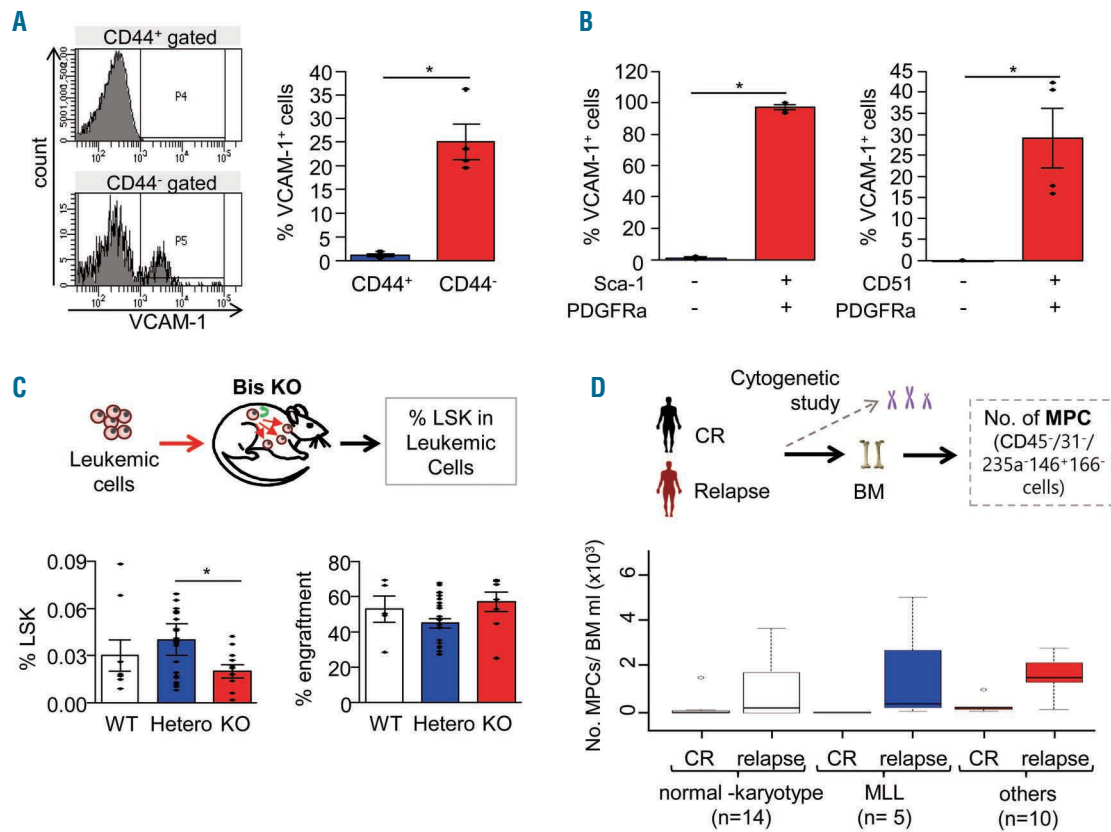
Figure 7. Dependency on the mesenchymal progenitor cells for generation of stem-like leukemic subsets. (A) Identification of VCAM-1-expressing mesenchymal stromal cells in the bone marrow (BM) of mice. % of VCAM-1(+) cells among murine mesenchymal cells (CD45-31-Ter119-) in fresh BM were compared for CD44(+) and CD44(-) cells. Shown are the representative flow cytometry plots (left) and quantification (right) for frequency of VCAM-1(+) cells among the indicated murine mesenchymal stromal subsets in fresh BM. (B) Comparisons of frequency of VCAM-1(+) cells in mice BM between the mesenchymal progenitor and non-progenitor subsets of murine mesenchymal stromal cells. Mesenchymal progenitor subsets in fresh mice BM were defined by PDGFRa(+)/Sca-1(+) or PDGFRa(+)CD51(+) based on published reports.[41-43,50] (C) Leukemogenesis in the Bis knockout (KO) mouse model. MN1 leukemic cells were transplanted into Bis KO mice, where mesenchymal progenitor populations are selectively decreased. Two weeks after transplantation into neonates of each mice model, engraftment of leukemic cells in BM and % LSK among engrafted leukemic cells were analyzed. Shown are the experimental design (upper) and % LSK leukemic subsets among engrafted leukemic cells for each indicated mice recipient (lower, left) and % engraftment of leukemic cells (GFP (+)) in BM (lower, right) (mean ± standard error of the mean [SEM], n=6 for wild-type [WT], n=27 for hetero, n=10 for KO). (D) Comparisons of mesenchymal progenitor cell numbers in BM of acute myeloid leukemia (AML) patients with respect to the clinical course. (upper) Experimental design. Fresh uncultured BM of AML patients without prior treatment were analyzed for cytogenetic abnormalities of leukemic blasts and content of mesenchymal progenitor cells (MPC; CD45-31-235a-146+166-) in fresh BM. Five years after the initial analysis, MPC numbers in patients' fresh BM were compared with subsequent clinical courses (maintenance of complete remission or relapse) with respect to the karyotype of leukemic blasts. (lower) Mean numbers of MPC (CD146+166-) in fresh BM of AML patients for each indicated clinical course and karyotype (mean ± SEM, n=14 for normal karyotype, n=5 for mixed-lineage leukemia [MLL], n=10 for others).

quiescence in cell cycle (*Online Supplementary Figure S8*)

In order to examine these findings in primary leukemic cells, we examined the response of primary AML blasts from five to seven individual patients to mesenchymal stroma. Primary AML blasts exhibited a significant induction of CD90(+) cells upon stromal contact, which was further increased by IL-4 treatment during co-culture (Figure 6C and D). Sort-purified subsets of CD90(+) and CD90(-) leukemic cells exhibited similar switching of phenotypes to maintain constant ratios in CD90(+) subsets in total leukemic cells, as observed for murine leukemic cells (Figure 6E). Moreover, the CD90 (+) subset generated during stromal contact exhibited higher resistance to Ara-C (Figure 6F) than the remaining CD90(-) cell population in the same co-culture, demonstrating a similar drug-resistance of newly emerging leukemic subsets in primary human leukemic cells.

Gene expression study on two independent patients showed that CD90(+) subsets of primary human AML cells are significantly enriched with gene sets specific for LSC[47]

than the remaining CD90(-) cells (Figure 6G), and enriched with gene sets involved in the interaction with the extracellular matrix (ECM) or focal adhesion (*Online Supplementary Figure S9*).

Thus, subsets of human leukemic cells in contact with stroma exhibit a stem-like properties to acquire drug-resistance through interaction with stroma.

Stromal heterogeneity for generation of stem cell-like leukemic subsets

Extensive heterogeneity has been documented among mesenchymal populations in BM stroma.[48,49] Therefore, we investigated the mesenchymal subpopulations responsible for generation of Sca-1(+) cells. Given that VCAM-1 expressing MSC played a role in the generation of drug-resistant subsets, we examined VCAM-1 expression among stromal cell populations in the BM. VCAM-1(+) mesenchymal cells were predominantly enriched by a CD44(-) population, where colony-forming mesenchymal progenitor cells (MPC) are exclusively localized[41-43,50] (Figure 7A).

Similarly, VCAM-1 is exclusively enriched in subsets for MPC as defined by PDGFRa(+)/Sca-1(+) or PDGFRa(+)/CD51(+) subsets,[41-43,50] indicating that the VCAM-1(+) cells that can drive emergence of drug-resistant subsets predominantly overlap with mesenchymal progenitor cells (Figure 7B).

Supporting this finding, when MN1 leukemic cells were transplanted into the homozygous Bis KO mice, where self-renewing MPC are decreased in the BM,[17,51] a significant decrease in the Sca-1(+) subset (LSK) among BM engrafted leukemic cells was observed compared to WT or heterozygote Bis KO mice (Figure 7C). This decrease was not associated with altered overall engraftment levels (Figure 7C), consistent with the differences between LSK and LK subsets.

Similarly, supporting the role of MPC for the development of drug resistant leukemic cells, AML patients wo relapsed after treatment exhibited higher numbers of mesenchymal progenitor subsets (MPC: CD146⁺/166⁻)[19, 43, 52] in BM, i.e., retrospective studies on AML patients who had undergone relapse within 1 year after complete remission exhibited higher numbers of mesenchymal progenitor subsets in the BM than those who maintained complete remission for 5 years (Figure 7D). Notably, this difference was observed regardless of the underlying cytogenetic abnormality of the leukemic blasts, indicating that the heterogeneity of stromal cells could be an additional factor drug-resistance of leukemic cells.

Altogether, this heterogeneity in the BM MPC can influence the stroma-dependent generation of stem cell-like leukemic subsets.

Discussion

Leukemic cell evolution has prevented the effective management of a diverse spectrum of leukemic disease.

Here, using a variety of murine and human leukemia cells both *in vitro* and *in vivo*, we show that subsets of leukemic cells can undergo a phenotypic conversion into a stem-like phenotype that exhibit a higher resistance to chemotherapy in the context of stromal contact. This development of chemoresistant subsets by stromal contact was not dependent on cell fusion or changes in leukemogenic activities observed in mitochondrial transfer.[27,28] The acquisition of the stem cell-like phenotype was reversible, being rapidly reverted to the non-stem cell phenotype under stroma-free conditions independent of difference in cell cycles or apoptosis (*Online Supplementary Figure S10*), unlike the stable maintenance of chemoresistance in leukemic clones generated by clonal evolution.

Moreover, the frequencies of LSK cells among leukemic cells in contact with stroma were maintained constant regardless of the phenotype of the initial cell populations. This suggests that the stroma-mediated development of the stem cell-like, drug-resistant subpopulation occurs in a stochastic and reversible manner in leukemic cells with similar probabilities among leukemic cells (equipotent) without clonal predisposition. Reminiscent of these findings, recent studies showed that non-stem cancer cells can be spontaneously converted to stem-like state, and these plasticity of cancer cells allows cellular switching between distinct functional states.[53,54] Together, these studies raise the possibility that the stochastic development of chemoresistant clones by stromal contact is an intrinsic process of leukemogenesis that could cause a non-

homogenous response to chemotherapy among the leukemic cell populations.

The mechanisms for dynamic equilibrium among different subsets of leukemic cells remains still unclear. One possibility is a feedback control mechanism that maintains a constant ratio of stem-like *versus* non-stem-like leukemic cells, probably through cellular interaction between distinct leukemic subsets, as inferred from clonal interactions between heterogenous subsets.[55] Similarly, studies on cancer stem cells have suggested that non-tumorigenic cells regulate the maintenance of cancer stem cells influencing their relative frequencies in the population.[56] Since clonal heterogeneity of leukemia or cancer cells underlies the differential response to chemotherapy and emergence of relapsing clones,[55,57,58] the kinetics of generating these stem-like subsets could be a factor for differential response to chemotherapy.

Interestingly, we show that the development of these drug-resistant leukemic subset is facilitated by bi-directional cross-talk between stroma and leukemic cells mediated by IL-4, exhibiting resistance to apoptosis (*Online Supplementary Figure S11*). While IL-4 was implicated in inhibition of leukemic cells and apoptosis,[59] we did not find increased apoptosis of the non-stem-like population precluding the selective enrichment of stem-like subsets by IL-4 (*Online Supplementary Figure S12*). Moreover, rather than acting directly on the leukemic cells, IL-4 targets stromal cells, which facilitate the generation of LSK subsets. How IL-4 acts on stromal cells to facilitate the generation of a drug-resistant leukemic subset remains unclear. However, we demonstrated a key role for VCAM-1 downstream of IL-4 in MSC leading to tight adherence of leukemic cells and MSC, which was necessary for generation of the LSK subset. Similarly, we found IL-4-dependent induction of gene clusters in MSC whose functions are related to 'binding function'. This suggests that the mode of interaction between MSC and leukemic cells is altered by IL-4 acting on MSC, which facilitates the development of the drug-resistant leukemic subset.

Interestingly, we also found functional differences between stromal cells in terms of their capacity to drive development of drug-resistant leukemic cells. We found that expression of VCAM-1 in stromal cell was important for adherence-dependent generation of leukemic subsets, while other adhesion molecules we tested did not influence the process (*Online Supplementary Figure S13*). Importantly, the VCAM-1-expressing stromal cells were selectively enriched in mesenchymal progenitors. Since mesenchymal stromal cells undergo various degenerative changes during leukemia,[19,60,61] it is possible that patient-to-patient heterogeneity in BM mesenchymal progenitor cell content could differentially contribute to the development of drug-resistant clones. Consistent with this, AML patients whose BM has higher levels of a primitive (CD146⁺) subset of mesenchymal cells[19,43,52] that express higher levels of VCAM-1 tend to have a higher risk of leukemic relapse compared to those who maintained complete remission. Thus, independent of oncogenic mutations or cytogenetic abnormalities in the blasts,[62] heterogeneity *per se* in mesenchymal progenitors in BM could be another factor for development of drug-resistant leukemic subsets.

In summary, our study reveals an additional mechanism of functional evolution of leukemic cells induced by contact with the mesenchymal stroma that can cause a reversible switch to a stem cell-like, drug-resistant subset independent

of mutation-driven clonal evolution in leukemic blasts (*Online Supplementary Figure S14*). These findings thus provide further insight into the multiple mechanisms for development of drug-resistance that could generate leukemic cells with distinct characteristics and chemoresistance, highlighting the importance of the microenvironment in this process. This supports the need for better defining the mechanisms of drug resistance in leukemia patients, and could lead to the development of more comprehensive management of leukemic diseases.

Disclosures

No conflicts of interest to disclose.

Contribution

HRL, GYL, EWK, HJK performed experiments and collected data, MHL performed experiments on genomics and statistical analysis of data; RHK conceptualized research, provided study

materials and wrote the manuscript; IHO conceptualized idea and research, supervised research, wrote the manuscript and provided financial support

References

1. Estey E, Dohner H. Acute myeloid leukaemia. Lancet. 2006;368(9550):1894-1907.
2. Craddock C, Tauro S, Moss P, Grimwade D. Biology and management of relapsed acute myeloid leukaemia. Br J Haematol. 2005;129(1):18-34.
3. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. Cell. 2012;150(2):264-278.
4. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-510.
5. Klco JM, Spencer DH, Miller CA, et al. Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. Cancer Cell. 2014;25(3):379-392.
6. Dick JE. Acute myeloid leukemia stem cells. Ann N Y Acad Sci. 2005;1044:1-5.
7. Hope KJ, Jin L, Dick JE. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nat Immunol. 2004;5 (7):738-743.
8. Eppert K, Takenaka K, Lechman ER, et al. Stem cell gene expression programs influence clinical outcome in human leukemia. Nat Med. 2011;17(9):1086-1093.
9. Guenechea G, Gan OI, Dorrell C, Dick JE. Distinct classes of human stem cells that differ in proliferative and self-renewal potential. Nat Immunol. 2001;2(1):75-82.
10. Ayala F, Dewar R, Kieran M, Kalluri R. Contribution of bone microenvironment to leukemogenesis and leukemia progression. Leukemia. 2009;23(12):2233-2241.
11. Konopleva M, Konoplev S, Hu W, Zaritskey AY, Afanasiev BV, Andreeff M. Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. Leukemia. 2002;16(9):1713-1724.
12. Katsumi A, Kiyoi H, Abe A, et al. FLT3/ITD regulates leukaemia cell adhesion through alpha4beta1 integrin and Pyk2 signalling. Eur J Haematol. 2011;86(3):191-198.
13. Ninomiya M, Abe A, Katsumi A, et al. Homing, proliferation and survival sites of human leukemia cells in vivo in immunodeficient mice. Leukemia. 2007;21(1):136-142.
14. Saito Y, Uchida N, Tanaka S, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. Nat Biotechnol. 2010;28(3):275-280.
15. Ishikawa F, Yoshida S, Saito Y, et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nat Biotechnol. 2007;25(11):1315-1321.
16. Doepfner KT, Boller D, Arcaro A. Targeting receptor tyrosine kinase signaling in acute myeloid leukemia. Crit Rev Oncol Hematol. 2007;63(3):215-230.
17. Youn DY, Lee DH, Lim MH, et al. Bis deficiency results in early lethality with metabolic deterioration and involution of spleen and thymus. Am J Physiol Endocrinol Metab. 2008;295(6):E1349-1357.
18. Kim IK, Kim BS, Koh CH, et al. Glucocorticoid-induced tumor necrosis factor receptor-related protein co-stimulation facilitates tumor regression by inducing IL-9-producing helper T cells. Nat Med. 2015;21(9):1010-1017.
19. Kim JA, Shim JS, Lee GY, et al. Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous leukemogenesis in acute myelogenous leukemia. Cancer Res. 2015;75(11):2222-2231.
20. Kim JH, Lee HS, Choi HK, et al. Heterogeneous niche activity of ex-vivo expanded MSCs as factor for variable outcomes in hematopoietic recovery. PloS One. 2016;11(12):e0168036.
21. Heuser M, Argiropoulos B, Kuchenbauer F, et al. MN1 overexpression induces acute myeloid leukemia in mice and predicts ATRA resistance in patients with AML. Blood. 2007;110(5):1639-1647.
22. Kroon E, Krosl J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J. 1998;17(13):3714-3725.
23. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31(2):166-169.
24. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26 (1):139-140.
25. Subramanian A, Kuehn H, Gould J, Tamayo P, Mesirov JP. GSEA-P: a desktop application for Gene Set Enrichment Analysis. Bioinformatics. 2007;23(23): 3251-3253.
26. Cogle CR, Goldman DC, Madlambayan GJ, et al. Functional integration of acute myeloid leukemia into the vascular niche. Leukemia. 2014;28(10):1978-1987.
27. Moschoi R, Imbert V, Nebout M, et al. Protective mitochondrial transfer from bone marrow stromal cells to acute myeloid leukemic cells during chemotherapy. Blood. 2016;128(2):253-264.
28. Wang J, Liu X, Qiu Y, et al. Cell adhesion-mediated mitochondria transfer contributes to mesenchymal stem cell-induced chemoresistance on T cell acute lymphoblastic leukemia cells. J Hematol Oncol. 2018;11(1):11.
29. Zuber J, Radtke I, Pardee TS, et al. Mouse models of human AML accurately predict chemotherapy response. Genes Dev. 2009;23(7):877-889.
30. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-737.
31. Kittang AO, Hatfield K, Sand K, Reikvam H, Bruserud O. The chemokine network in acute myelogenous leukemia: molecular mechanisms involved in leukemogenesis and therapeutic implications. Curr Top Microbiol Immunol. 2010;341:149-172.
32. Hassan HT, Zander A. Stem cell factor as a survival and growth factor in human normal and malignant hematopoiesis. Acta Haematol. 1996;95(3-4):257-262.
33. Mazur G, Wrobel T, Butrym A, Kapelko-Slowik K, Poreba R, Kuliczkowski K. Increased monocyte chemoattractant protein 1 (MCP-1/CCL-2) serum level in acute myeloid leukemia. Neoplasma. 2007;54(4): 285-289.
34. Yang J, Liu X, Nyland SB, et al. Platelet-derived growth factor mediates survival of leukemic large granular lymphocytes via an autocrine regulatory pathway. Blood. 2010;115(1):51-60.
35. Akashi K, Harada M, Shibuya T, et al.

Effects of interleukin-4 and interleukin-6 on the proliferation of CD34+ and CD34- blasts from acute myelogenous leukemia. Blood. 1991;78(1):197-204.

36. Piccaluga PP, Rossi M, Agostinelli C, et al. Platelet-derived growth factor alpha mediates the proliferation of peripheral T-cell lymphoma cells via an autocrine regulatory pathway. Leukemia. 2014;28(8):1687-1697.

37. Tsuyada A, Chow A, Wu J, et al. CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. Cancer Res. 2012;72(11):2768-2779.

38. Ding W, Knox TR, Tschumper RC, et al. Platelet-derived growth factor (PDGF)-PDGF receptor interaction activates bone marrow-derived mesenchymal stromal cells derived from chronic lymphocytic leukemia: implications for an angiogenic switch. Blood. 2010;116(16):2984-2993.

39. Schulz A, Toedt G, Zenz T, Stilgenbauer S, Lichter P, Seiffert M. Inflammatory cytokines and signaling pathways are associated with survival of primary chronic lymphocytic leukemia cells in vitro: a dominant role of CCL2. Haematologica. 2011;96(3):408-416.

40. Juneja HS, Schmalsteig FC, Lee S, Chen J. Vascular cell adhesion molecule-1 and VLA-4 are obligatory adhesion proteins in the heterotypic adherence between human leukemia/lymphoma cells and marrow stromal cells. Exp Hematol. 1993;21(3):444-450.

41. Morikawa S, Mabuchi Y, Kubota Y, et al. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. J Exp Med. 2009;206(11):2483-2496.

42. Pinho S, Lacombe J, Hanoun M, et al. PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. J Exp Med. 2013;210(7):1351-1367.

43. Qian H, Le Blanc K, Sigvardsson M. Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. J Biol Chem. 2012;287(31):25795-25807.

44. Peault B, Weissman IL, Buckle AM, Tsukamoto A, Baum C. Thy-1-expressing CD34+ human cells express multiple hematopoietic potentialities in vitro and in SCID-hu mice. Nouv Rev Fr Hematol. 1993;35(1):91-93.

45. Buccisano F, Rossi FM, Venditti A, et al. CD90/Thy-1 is preferentially expressed on blast cells of high risk acute myeloid leukaemias. Br J Haematol. 2004;125(2):203-212.

46. Yamazaki H, Nishida H, Iwata S, Dang NH, Morimoto C. CD90 and CD110 correlate with cancer stem cell potentials in human T-acute lymphoblastic leukemia cells. Biochem Biophys Res Commun. 2009;383(2):172-177.

47. Saito Y, Kitamura H, Hijikata A, et al. Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. Sci Transl Med. 2010;2(17):17ra19.

48. Oh IH, Kwon KR. Concise review: multiple niches for hematopoietic stem cell regulations. Stem Cells. 2010;28(7):1243-1249.

49. Wei Q, Frenette PS. Niches for hematopoietic stem cells and their progeny. Immunity. 2018;48(4):632-648.

50. Mendez-Ferrer S, Michurina TV, Ferraro F, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature. 2010;466(7308):829-834.

51. Kwon KR, Ahn JY, Kim MS, Jung JY, Lee JH, Oh IH. Disruption of bis leads to the deterioration of the vascular niche for hematopoietic stem cells. Stem Cells. 2010;28(2):268-278.

52. Sacchetti B, Funari A, Michienzi S, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell. 2007;131(2):324-336.

53. Chaffer CL, Brueckmann I, Scheel C, et al. Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. Proc Natl Acad Sci U S A. 2011;108(19):7950-7955.

54. da Silva-Diz V, Lorenzo-Sanz L, Bernat-Peguera A, Lopez-Cerda M, Munoz P. Cancer cell plasticity: impact on tumor progression and therapy response. Semin Cancer Biol. 2018;53:48-58.

55. McGranahan N, Swanton C. Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. Cancer Cell. 2015;27(1):15-26.

56. Sprouffske K, Athena Aktipis C, Radich JP, Carroll M, Nedelcu AM, Maley CC. An evolutionary explanation for the presence of cancer nonstem cells in neoplasms. Evol Appl. 2013;6(1):92-101.

57. Jamal-Hanjani M, Quezada SA, Larkin J, Swanton C. Translational implications of tumor heterogeneity. Clin Cancer Res. 2015;21(6):1258-1266.

58. Quek L, David MD, Kennedy A, et al. Clonal heterogeneity of acute myeloid leukemia treated with the IDH2 inhibitor enasidenib. Nat Med. 2018;24(8):1167-1177.

59. Peña-Martínez P, Eriksson M, Ramakrishnan R, et al. Interleukin 4 induces apoptosis of acute myeloid leukemia cells in a Stat6-dependent manner. Leukemia. 2018;32(3):588-596.

60. Schepers K, Pietras EM, Reynaud D, et al. Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. Cell Stem Cell. 2013;13(3):285-299.

61. Zhang B, Ho YW, Huang Q, et al. Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia. Cancer Cell. 2012;21(4):577-592.

62. Byrd JC, Mrozek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002;100(13):4325-4336.