Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous 
leukemogenesis in acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous disorder characterized by clonal proliferation of stem cell-like blasts in bone marrow; however, their unique cellular interaction within the bone marrow microenvironment and its functional significance remain unclear. Here, we assessed the bone marrow microenvironment of AML patients and demonstrate that the leukemia stem cells induce a change in the transcriptional programming of the normal mesenchymal stromal cells. The modified leukemic niche alters the expressions of cross-talk molecules (i.e., CXCL-12 and Jagged-1) in mesenchymal stromal cells to provide a distinct cross-talk between normal and leukemia cells, selectively suppressing normal primitive hematopoietic cells while supporting leukemogenesis and chemoresistance. Of note, AML patients exhibited distinct heterogeneity in the alteration of mesenchymal stroma in bone marrow. The distinct pattern of stromal changes in leukemic bone marrow at initial diagnosis was associated with a heterogeneous post-treatment clinical course with respect to the maintenance of complete remission for 5-8 years and early or late relapse. Thus, remodeling of mesenchymal niche by leukemia cells is an intrinsic self-reinforcing process of leukemogenesis that can be a parameter for the heterogeneity in the clinical course of leukemia and hence serve as a potential prognostic factor.
INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells characterized by excessive proliferation of stem cell-like progenitor cell in bone marrow (BM) with a heterogeneous prognosis (1). Xenotransplantation studies of AML demonstrate a hierarchical organization of AML and identified a subpopulation of leukemia cells called leukemic stem cells (LSCs); they arise from the transformation of hematopoietic stem cells (HSCs) or re-acquisition of self-renewal capability in committed progenitors (2,3) and can initiate and maintain their leukemic state. As LSCs share stem cell-like properties with normal HSCs, LSCs may be regulated within the BM microenvironment (4).

The BM microenvironment for HSC is characterized by a specialized architecture termed the stem cell niche localized in the perivascular or endosteal area of BM where most HSCs reside(5). While the niche is comprised of various types of stromal cells from mesenchymal and non-mesenchymal origin (6-8), a subset of mesenchymal stromal cells (MSCs) that retain colony-forming potential (CFU-F) and self-renewal ability could reconstitute both types of niches in the heterologous marrow model (9,10). Subsequent studies identified additional subsets of mesenchymal cells including early-stage osteoblastic cells expressing Runx2 (11),nestin (12) or leptin-receptor (13), or primitive (prx-1+) MSCs (14) as major regulators of HSCs in the BM niche. These niche cells express cross-talk molecules such as Jagged-1(15,16), CXCL-12 (14,17,18), and angiopoietin-1 (19) to interact with HSCs to regulate their self-renewal (15,20), quiescence (21-23), and mobilization(24).

The LSCs, when transplanted, engraft in the BM like normal HSCs, competing for niche with normal HSCs (25) and influence the normal stem cell niche, causing alteration in the microenvironmental regulation. For example, leukemia cells transplanted into mice create an abnormal BM niche to usurp the transplanted normal HSCs into a tumor niche (26). In addition, a transgenic model of chronic myeloid leukemia shows defective homing and retention of HSCs in the niche due to decreased CXCL12 in BM MSCs (27). Similarly, mice with bcr-abl–transformed leukemia exhibit analogous alterations of MSCs and osteoblastic cells along with their supporting effects on leukemogenesis (28,29), thus giving rise to the concept of the “leukemic niche” (30,31).
Nevertheless, little is known about the leukemic microenvironment in human leukemic diseases or its clinical relevance to the leukemogenic process. Here, using a human AML model, we report the leukemic alterations of the microenvironment in AML patient’s BM and their impact on the clinical course of AML patients.

MATERIALS AND METHODS

Study design and sample collection

For the initial screening of BM stroma in AML, 51 BM samples from treatment-naïve AML patients were randomly collected. For the cohort study on prognosis, another set of BM samples was collected from 48 patients newly diagnosed with AML without prior treatment history who had complete medical records during 5–8 years of follow-up. This study was approved by the Institutional Review Boards of St. Mary’s Hospital and Seoul St. Mary’s Hospital. All samples including umbilical cord blood cells were obtained after obtaining informed consent.

Analysis of BM stromal cells

The in vitro culture of MSCs and colony forming assay (CFU-F) were performed as described previously (32). Senescence-associated β-galactosidase (SA-β-gal) activity was examined by a senescence β-galactosidase staining kit (Cell Signaling, Danvers, MA, USA). Specific subsets of MSCs in fresh bone marrow mononuclear cells were analyzed by flow cytometry after staining with specific antibodies against MSC markers (Supplementary Table S1 for antibodies). Cross-talk molecule expression on MSCs was similarly analyzed by flow cytometry after staining against Jagged-1 and CXCL-12 as described previously (16,33) (see Supplementary Table S1 for antibodies).

Ex vivo culture of normal hematopoietic and leukemia cells

MSCs were irradiated with 1,500 cGy 24 hours prior to co-culture with normal or leukemic CD34+
cells for 5 days in long-term culture media (H5100, STEMCELL Technologies, Vancouver, Canada) in the presence of a cytokine mixture (100 ng/mL human SCF; 100 ng/mL human Flt3L; and 20 ng/mL human IL-3, IL-6, and G-CSF; ProSpec-Tany TechnoGene Ltd., Rehovot, Israel). For long-term culture-initiating cell (LTC-IC) analysis, CD34+ cells were co-cultured with normal MSCs or AML-derived MSCs (AML-MSCs) for 5 days, transferred to a 6-week long-term culture, and subjected to a colony-forming assay in semi-solid medium (34).

**Animal leukemia transplantation model**

BM progenitor cells from C57BL6 mice were transformed to leukemia cells (AML) by transduction of MN1 as described (35) and transplanted into congeneic mice to establish transplantation leukemia model. Repopulating assay in neonatal mice disrupted with bis (Bis-knockout) were performed as described (33).

**NOD/SCID-γnull repopulation assay**

NOD/SCID-γnull mice were irradiated with 250 cGy and intravenously injected with hematopoietic cells co-cultured on MSCs. Human cell engraftment was analyzed as described previously (36).

**Gene expression analysis of MSCs**

MSC gene expression was analyzed by Illumina BeadChip (Illumina, San Diego, CA) array hybridization analysis, using the Linear Models for Microarray Data (LIMMA) method and the R-package for Statistics as described previously (37). For expression profiles, the median absolute deviation was calculated, and highly variable genes were selected for hierarchical clustering with average linkage. Gene set enrichment analysis was performed using the GO categories from MSigDB (PMID: 16199517).

**Statistical analysis**

For gene set enrichment analysis, normal $P$-values of significance were estimated for 1,000
permutations of genes for each GO category to select significant GO categories (i.e., false discovery rate $< 0.1$). The predictive performance of each stromal cell parameter for leukemic relapse was determined by receiver operator characteristic (ROC) curve analysis and by calculating the areas under curve (AUCs) with standard errors. All data were analyzed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA), and the level of significance was set at $P < 0.05$.

RESULTS

Altered mesenchymal cells in BM in AML

To investigate the characteristics of the BM microenvironment specific to human leukemic conditions, we first analyzed the mesenchymal cellular composition of the fresh BM stromal cells in treatment-naïve patients initially diagnosed with AML. Compared to normal BM, the frequency of primitive subsets (CD146+166-) of MSCs (10,38,39) was significantly lower, whereas the frequency of mature osteoblastic cells (CD146-166+) (40) was higher (Fig. 1A), indicating an overall shift in mesenchymal differentiation. We subsequently examined the colony formation of MSCs in normal and leukemic BM, because niche cells are enriched in colony-forming mesenchymal progenitors (9,10). Compared to normal BM, BM mononuclear cells from AML patients frequently failed to form colonies, and exhibited a higher frequency of accelerated growth arrest (within 2 passages of subculture) and higher senescence-associated $\beta$-galactosidase activity (Fig. 1B, C, Supplementary Fig. S1A). Even among AML-MSCs that continued to grow beyond 3 passages, decreased proliferation was observed during the 60-day subculture (Supplementary Fig. S1B). Altered MSC function was observed in AML patients irrespective of AML subtype (Supplementary Fig. S1A). Of note, while the overall numbers of CFU-Fs were lower in AML BM than age-matched normal BM, no difference was observed when AML patients who achieved complete remission (Fig. 1D, E), indicating that mesenchymal alterations reflect ongoing leukemogenic activities in AML BM.

To determine if these mesenchymal alterations can be caused by leukemic blasts, we examined MSCs
co-cultured with leukemic blasts and compared their transcription profiles with those of MSCs co-cultured with normal hematopoietic progenitors (Fig. 2A). Hierarchical clustering of highly variable genes clearly segregated the MSCs co-cultured with normal and leukemic blasts (Fig. 2B), indicating a substantial difference in transcriptomes. Gene set enrichment analysis was performed to identify the candidate molecular functions associated with these transcriptomic changes. Overall, 11 and 80 GO categories exhibited significant enrichment with upregulated and downregulated genes, respectively, in MSCs co-cultured with leukemic CD34+ cells compared with those co-cultured with normal CD34+ cells (Fig. 2C, Supplementary Table S2). Of note, among GO categories downregulated under leukemic conditions, genes for “cell-cycle” and related functions (e.g., “chromosome” and “DNA replication”) were significantly enriched, consistent with the loss of proliferation in AML-MSCs. The enrichment plot of 105 genes from “cell cycle”-related gene sets is shown with the top 20 leading-edge genes (Fig. 2D, Supplementary Table S3). In contrast, among the GO categories upregulated in leukemia-cultured MSCs, 2 cytokine-related GO functions were observed: “chemokine receptor binding” and “chemokine activity” (Fig. 2C, Supplementary Fig. S2). These results indicate leukemia cells can indeed induce transcriptomic reprogramming of MSCs distinct from normal hematopoietic cells with prominent suppression of cell cycle-related genes and upregulation of cytokine-related genes.

The leukemic niche resets microenvironmental cross-talk for normal and leukemia cells

Leukemia-induced mesenchymal alterations were further examined with respect to changes in the expressions of major cross-talk molecules, Jagged-1 and CXCL-12, which regulate HSCs in the BM niche (5,15,16). First, the mesenchymal expression of Jagged-1, was examined during co-culture with normal or leukemia cells (Fig. 3A). Co-culture with normal hematopoietic progenitors increased and sharply decreased the percentage of Jagged-1(+) cells in normal MSCs and AML-MSCs, respectively, demonstrating distinct responses in these MSCs (Fig. 3B). Accordingly, normal hematopoietic progenitors co-cultured with AML-MSCs exhibited significantly suppressed downstream notch signals Hes-1 and Hes-5 compared to co-culture with normal MSCs (Fig. 3C). In contrast, co-culture...
with leukemic blasts did not alter the percentages of Jagged-1(+)-cells in normal MSCs or AML-MSCs (Fig. 3D). These results indicate Jagged-1 cross-talk is selectively suppressed for normal hematopoietic progenitors but maintained for leukemic blasts on AML-MSCs. Likewise, the percentage of CXCL-12(+) cells among co-cultured MSCs increased markedly and selectively in response to leukemia cells but decreased during co-culture with normal hematopoietic cells (Fig. 3E). Moreover, the fresh uncultured BM stromal cells from AML patients exhibited higher percentage of CXCL-12(+) MSCs than in normal BM, indicating similar alterations of cross-talk under in-vivo condition. (Fig. 3F, Supplementary Fig. S3). These results indicate the microenvironment altered by leukemia cells differentially responds to normal and leukemia cells to reset the cross-talks among normal and leukemia cells.

**Altered microenvironments provide a selective advantage to leukemia cells over normal HSCs**

Next, we investigated the functional impacts of leukemic MSCs on normal hematopoietic function by co-culturing normal CD34+ cells with normal MSCs or AML-MSCs (Figs. 4A, 5A). Significant ex vivo expansion of CD34+ cells was observed in co-culture with normal MSCs but not with AML-MSCs (Fig. 4B). Similarly, when transplanted into NOD/SCID-ɤcnull mice (41), normal hematopoietic progenitors co-cultured on AML-MSCs exhibited lower repopulating activities than cells co-cultured with normal MSCs (Fig. 4C). In particular, analysis of HSCs by LTC-IC assay(42) showed a profound loss of LTC-ICs in CD34+ cells co-cultured with AML-MSCs, sharply contrasting with their maintenance with normal MSCs (Fig. 4D). These results demonstrate AML-MSCs suppress normal hematopoiesis and that primitive hematopoietic cells are most profoundly affected by the deteriorating effects of leukemic MSCs.

In contrast, when various types of leukemia cells were co-cultured with normal MSCs or AML-MSCs, there were no differences in proliferation in vitro (Fig. 5B). Similarly, there was no difference in leukemogenesis in vivo between NOD/SCID-ɤcnull mice transplanted with leukemia cells co-cultured with normal MSCs or AML-MSCs (Fig. 5C, Supplementary Fig. S4), indicating resistance of leukemia cells to the detrimental niche. Moreover, co-culture with AML-MSCs drove greater
proportions of leukemia cells into quiescence and conferred greater resistance to Ara-C–induced apoptosis than co-culture with normal MSCs (Fig. 5D, E).

These results collectively demonstrate that the leukemic microenvironment distinctively affects normal and leukemia cells, selectively suppressing normal hematopoietic cells but supporting the leukemogenic activity and chemoresistance of leukemia cells.

**Animal leukemia transplantation model**

Given the characteristics of the leukemic niche in human AML, we determined if they can be reproduced in an animal model engrafted with leukemia cells. Thus, we established a mouse leukemia model for AML by transplanting MN-1-induced leukemia(43) (Fig. 6A). Examination of the BM microenvironment showed a profound loss of MSCs and decreased self-renewal of mesenchymal progenitor cells to produce secondary CFU-Fs (Fig. 6B, C). We subsequently investigated the engraftment advantage of leukemia cells over normal hematopoietic cells in deteriorated mesenchymal niche. To this end, we employed a Bis-knockout mouse model, where the mesenchymal niche of BM was deteriorated causing a microenvironmental defect in their hematopoiesis (33) (Fig. 6D). When normal hematopoietic or leukemia cells (MN-1) were transplanted into Bis-knockout mice, normal hematopoietic cell engraftment was lower in Bis-knockout mice than wild-type mice (Fig. 6E). However, leukemia cells exhibited comparable engraftment in Bis-knockout and wild-type mice, indicating the leukemogenic process is more resistant to the deterioration of the mesenchymal niche (Fig. 6F). These results collectively indicate the alteration of the mesenchymal niche by leukemia cells is an intrinsic process of leukemogenesis and that leukemia cells can have a selective advantage in this altered microenvironment.

**Stromal remodeling as a factor in the heterogeneous clinical course of leukemia patients**

Given that leukemic stromal remodeling functionally influences normal hematopoietic and leukemogenic activities, we hypothesized that differences in the pattern of stromal alteration are an underlying factor of the heterogeneity of the clinical course of AML patients. Accordingly, we
examined the association between the BM stromal changes at initial AML diagnosis and subsequent clinical course for 5–8 years after remission. Stromal cell composition in BM biopsy taken at initial diagnosis was examined in AML patients who maintained complete remission for 5–8 years, relapsed after remission, and exhibited a refractory response to chemotherapy along with BM from normal donors (see Supplementary Table S4 for patients).

While individual AML patients exhibited heterogeneity in stromal cell composition, there were substantial differences in the BM content of mesenchymal cellular components, but not endothelial cells, among the AML patients who maintained complete remission and those relapsed after remission; the BM of the relapse group exhibited more MSCs, osteoblastic cells (OB), CFU-Fs and primitive MSCs (P-MSCs)(CD146+CD45-31-235a-166-), a subpopulation of MSCs enriched with nestin+ cells (Supplementary Fig. S5), than complete remission group (Supplementary Fig. S6A, B).

Therefore, to determine if such differences in BM stromal cell patterns at the initial diagnosis of AML can identify patients with a high risk of relapse, we performed ROC curve analysis of each stromal component and compared their prediction performance according to the AUCs.

The AUCs for the prediction of total relapse according to the numbers of MSCs (0.78), P-MSCs (0.72), and OBs (0.70) were moderately higher than those according to the numbers of endothelial cells (0.63) (Fig. 7A). However, the prediction for relapse became stronger when early (≤1 year) and late (>1 year) relapse were analyzed separately; early relapse (≤1 year) was associated with greater P-MSC content in BM and with significant predictability (AUC, 0.8 ± 0.08), which further strengthened for relapse within 6 months after remission (AUC, 0.88 ± 0.06) (Fig. 7B). In contrast, patients with late relapse exhibited significantly more MSCs or OBs but not P-MSCs in BM than in those with complete remission, with high predictabilities for late relapse (AUC, 0.91 ± 0.06 and 0.88 ± 0.08 for MSCs and OBs, respectively) (Fig. 7B). These results indicate early and late relapse of AML are associated with distinct stromal microenvironments, i.e., large numbers of P-MSCs in BM is strongly associated with early relapse, whereas large numbers of MSCs or OBs is significantly associated with late relapse.

These findings collectively suggest differences in stromal patterns in leukemic BM at initial diagnosis.
are associated with different clinical courses in AML patients. (Fig. 7C).

**DISCUSSION**

The notion that leukemic stem cells share the stemness of normal HSCs prompted studies on the cellular interaction between leukemia cells and the BM microenvironment. Recently, studies using animal leukemia models reported various types of changes in the bone marrow niche cells (27,29,44). Similarly, changes in OVs or MSC numbers were observed in a limited cases of leukemia patients (45,46). However, systemic analysis of the microenvironmental alterations in various leukemic conditions have not been made, nor their relevance to clinical courses. Our initial analysis of samples from AML patients revealed a significant loss of primitive MSC populations, which is associated with loss of MSC colonization and self-renewal. In addition, while normal HSCs was shown to induce transcriptional changes in MSCs (47), leukemic blasts reprogram the transcriptomes of MSCs distinct from normal hematopoietic cells, profoundly downregulating cell cycle-promoting genes and upregulating cytokine-related genes. The altered MSCs also reset the niche cross-talks by distinctively altering expression of cross-talk molecules such as Jagged-1 and CXCL-12 for normal and leukemia cells. Accordingly, leukemic MSCs selectively suppress normal hematopoietic functions while sparing the leukemogenic activity and chemoresistance of leukemia cells, thereby contributing to the clonal dominance of leukemia cells over their normal hematopoietic counterparts in the BM. In particular, LTC-ICs, the primitive hematopoietic subpopulation analogous to in vivo long-term repopulating cells (48), were most profoundly lost during co-culture with AML-MSCs; in contrast, their repopulation is less severely affected in NOD/SCID-γcnull mice, into which both long- and short-term hematopoietic progenitors can be engrafted (49). Thus, primitive hematopoietic progenitors appear to be more vulnerable to the deteriorating effects of leukemic MSCs. Corroborating this notion, AML is frequently associated with a loss of multi-lineage hematopoiesis, including anemia, as well as a loss of leukocytes or platelets as a major cause of morbidity. Hence, the leukemia-induced
remodeling of the niche resulting in a deteriorated microenvironment can be viewed as another pathogenic mechanism of leukemic diseases.

Interestingly, the alteration of the mesenchymal niche was reproduced in the transplantation leukemia model in mice, which exhibited a marked loss of mesenchymal stroma and self-renewal of mesenchymal progenitors. Furthermore, the advantage of leukemia cells over normal hematopoietic cells under such a deteriorated microenvironment was also observed in the mouse model with defective mesenchymal niche in BM, the Bis-knockout model. Thus, the leukemia-induced remodeling of the mesenchymal niche, which distinctly influence normal and leukemia cells, appears to be an intrinsic pathogenic process of leukemogenesis in both animal and clinical models.

Of note, while leukemia cells induce various patterns of stromal alterations in BM, the mechanisms of various stromal changes in AML patients remain unclear. Recent animal studies demonstrate that excessive cytokine secretion, which also differs with respect to specific oncogenic mechanisms, is a force driving mesenchymal niche alterations (27-29). The present study revealed that the genes upregulated in leukemia-cultured MSCs were highly enriched among GO categories related to “chemokine activity” implicating similar involvement of excessive cytokine activity in this process. However, studies characterizing the mechanisms underlying heterogeneity in stromal remodeling in each AML patient as well as expression studies under in-vivo conditions to overcome potential discrepancies from in-vitro conditions are warranted.

Nevertheless, the present study demonstrates the clinical significance of stromal remodeling as an intrinsic process of self-reinforcing leukemogenesis and factor underlying the heterogeneous clinical course in AML patients; thus, the difference in stromal cell components in BM at initial diagnosis is closely associated with the clinical course, particularly the maintenance of complete remission for 5–8 years or relapse after remission during follow-up. The reason for the observed differences in leukemogenesis with respect to stromal environment is unclear. However, recent studies demonstrate heterogeneous subtypes of MSCs in BM exhibit different niche function and that the primitive subsets of MSCs play a dominant role in the maintenance and self-renewal of HSCs (14). Therefore, heterogeneity in subpopulations of MSCs in BM may provide different niche environments for
leukemia stem cells, leading to heterogeneous kinetics of leukemia relapse in each distinct stromal environment. In this case, it is plausible that a lack of P-MSCs in BM would preclude the maintenance and self-renewal of the LSCs responsible for relapse, leading to the maintenance of complete remission; meanwhile, high levels of P-MSCs or MSCs/osteoblastic cells would support the maintenance and/or self-renewal of LSCs in a distinct manner (Fig. 7C).

Thus, the present findings indicate the patterns of BM stromal changes at initial diagnosis of AML may predict patients with a high risk of relapse. However, while our study provides insights regarding the clinical relevance of stromal changes for leukemogenesis as a hypothesis-generating study, its application as a clinical biomarker requires additional cohort studies with larger sample sizes as a “hypothesis-testing” study.

In conclusion, the present study demonstrates that the leukemia-induced remodeling of the BM microenvironment is an intrinsic part of leukemogenesis and may be responsible for the heterogeneity of the clinical course of AML and hence a potential prognostic parameter in AML.

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FIGURE LEGENDS

Figure 1. Altered mesenchymal stroma in the BM of AML patients

(A) Comparison of mesenchymal cell composition in BM. Percentages of primitive MSCs (CD45-31-235a-146+166-) and differentiated osteoblastic cells (CD45-31-235a-146-166+) in the total MSC population (CD45-31-235a-) in fresh BM (mean ± SEM, n = 51, 11 for AML and normal BM, respectively). (B) Colony-forming activity (CFU-F) and proliferation of MSCs in AML and normal BM. The percentage of BMs that did not form any CFU-F colonies (no colony), formed colonies but reached growth arrest within 2 passages of subculture (growth arrest), and continued proliferating beyond 2 passages (normal growth) are shown (n = 51 and 11 for AML and normal BM, respectively). (C) Senescence-associated β-galactosidase activity of AML and normal BM-derived MSCs. Mean ± SEM of 3 independent experiments (n = 9 and 12 for normal and AML, respectively). (D) CFU-Fs in the BM of normal and age-matched AML patients at initial diagnosis. Box plot shows CFU-Fs obtained from mononuclear cells contained in 1 mL BM with mean ages (n = 11 and 51, for normal and AML, respectively) (*P < 0.05). (E) CFU-Fs after AML remission. CFU-Fs in equivalent numbers of BM mononuclear cells (5 × 10⁶) were plated to compare normal and hypocellular BM after AML remission (n = 11 and 17 for normal and AML-remission, respectively) (P > 0.05).

Figure 2. Transcription profiles of MSCs co-cultured with AML blasts or normal hematopoietic cells

(A) Experimental design schematic (n = 3 per group). (B) Hierarchical clustering of 1,000 variable probes (median absolute deviation > 0.04) segregates the MSCs co-cultured with normal and leukemic CD34+ cells. (C) Top 10 significant GO categories upregulated and downregulated in MSCs co-cultured with leukemic CD34+ cells vs. normal CD34+ cells; numbers of genes for each GO category are shown in parentheses (Supplementary Table 2 for the full list of 11 and 80 upregulated and downregulated GO categories, respectively). (D) Gene set enrichment plot. All genes downregulated in MSCs co-cultured with leukemia cells are sorted by differential expression. The locations of 105 genes collected from 10 “cell-cycle”-related GO categories are shown with the top 20
leading-edge genes listed below (see Supplementary Table S3 for the full list of the top 20 leading-edge genes).

**Figure 3. Alteration of cross-talk molecules in MSCs co-cultured with normal hematopoietic or leukemia cells**

(A) Experimental design schematic. MSCs culture-derived from normal or AML patients (AML-MSCs) were co-cultured with normal or leukemic for 5 days, and cross-talk molecules in MSCs were examined. (B) Percentages of MSCs expressing Jagged 1 protein determined by flow cytometry ($n = 4$ per group, 4 experiments) ($^*P < 0.05$, $^{***}P < 0.001$). Mean percentages are pooled normal and AML (M1, M2, M5a) groups. (C) Effects of normal and AML-MSCs on downstream notch signals in co-cultured hematopoietic progenitors. Mean ± SEM folds of transcripts in normal CD34+ cells co-cultured with indicated individual MSCs relative to levels in stroma-free (SF) conditions are shown (3 experiments, $n = 3$ per group). (D) Mean ± SEM of MSCs expressing Jagged-1 protein in the presence or absence of leukemic CD34+ cells determined by flow cytometry (3 experiments, $n = 3$ per group). (E) Changes in CXCL-12 expression in normal MSCs or AML-MSCs co-cultured with normal or leukemic blasts. Mean ± SEM percentages of CXCL-12(+) cells in each group of MSCs are shown (3 experiments, $n = 3$ per group) ($^*P < 0.05$, $^{**}P < 0.01$). (F) In vivo expression of CXCL-12 in fresh uncultured stromal cells in BM from normal donors and AML patients. Mean ± SEM percentages of CXCL-12 (+) cells among total MSCs (CD45-31-235a-) in fresh BM are shown ($n = 8$ and 46 for normal and AML, respectively).

**Figure 4. Functional impact of leukemic MSCs on normal hematopoietic cells**

(A) Experimental design schematic. (B) Ex vivo expansion of normal CD34+ cells ($1 \times 10^5$ input CD34+ cells) during stroma-free (SF) or co-culture on each type of MSC (3 experiments, $n = 7$ per group) ($^*P < 0.05$). (C) Engraftment of co-cultured normal CD34+ cells in individual mice. Normal CD34+ cells co-cultured with each group of MSCs were transplanted into irradiated NSG mice ($1 \times 10^4$ input CD34+ cells/mouse). The percentage engraftment of human CD45+ cells 8 weeks post-
transplantation and frequency of positive engraftment (>1%) of all test mice are shown (left) with mean ± SEM engraftment levels in each normal and leukemic MSC group (right) (*P < 0.05). (D) Effects of normal and AML-MSCs on LTC-ICs. Normal CD34+ cells (400 input cells) co-cultured on the indicated MSCs for 5 days were subjected to long-term culture assay for 6 weeks followed by plating on the semisolid medium. Numbers of colonies derived from long-term cultures are shown (4 experiments, n = 4 per group).

Figure 5. Functional influence of AML-MSCs on leukemogenesis

(A) Experimental design schematic. (B) Effects of normal or AML-MSCs on the proliferation of AML blasts (M1, M3, HL-60) during co-culture for 5 days (3 experiments, n = 5-6 for each group). (C) Mean ± SEM percentage of engraftment of leukemia cells (HL-60) co-cultured for 5 days on each group of MSCs in peripheral blood (PB) and BM of NSG recipient mice (n = 6 per group) (Supplementary Figure S4 for the engraftment of primary leukemia cells). (D) Cell cycling of leukemia cells during co-culture analyzed by staining with Hoechst 33342 and pyronin Y. The mean ± SEM percentages of leukemia cell populations (HL-60) in the G0 phase are shown with representative plots (2 experiments, n = 2 for SF and n = 5–6 for co-culture, *P < 0.05). (E) Apoptosis of leukemia cells (HL-60) induced by Ara-C treatment (2 μM, 2 days) during co-culture. The mean ± SEM of apoptotic cells (Annexin V+PI-) are shown (3 experiments, n = 3 and 5–6 for SF and co-culture, respectively).

Figure 6. Alteration of mesenchymal niche in the animal transplantation leukemia model and effects on leukemogenesis

(A) Schematic of the animal transplantation leukemia model established by transplanting MN1-induced AML cells. Representative flow cytometry plots for leukemic engraftment and BM section are shown. (B) Total number of MSCs (CD45-31-Ter119-) in BMs of mice transplanted with media (HF2), normal BM cells, or AML cells (MN1) (3 experiments, n = 8). (C) Self-renewal of mesenchymal progenitors determined by the number of secondary CFU-Fs from each primary CFU-F
(3 experiments, n = 8). (D) Schematic of experimental design to compare the influence of defective mesenchymal niche on the engraftment of normal and leukemia cells. (E) Numbers of donor-derived normal hematopoietic cells engrafted in each neonatal wild-type or Bis knockout(KO) mice (5 experiments, n = 12). (F) Numbers of MN1-AML cells engrafted in neonatal wild-type or Bis knockout mice (5 experiments, n = 10).

**Figure 7. Association between BM stromal changes at initial diagnosis and the subsequent clinical course of AML**

AML patients were grouped into those who maintained complete remission for 5–8 years (CR, n = 29) and those who relapsed after remission (R, n = 14); BM samples taken at initial diagnosis were compared with respect to the numbers of MSCs (CD45-31-235a-) (MSCs), endothelial cells (CD45-31-235a-31+) (ECs), primitive MSCs (CD45-31-235a-146+166-) (P-MSCs), mature osteoblastic cells (CD45-31-235a-146-166+) (OBs), and colony-forming cells (CFU-F) contained in 1 mL fresh BM (Supplementary Figure S6 for box-plot data). (A) Predictive performance of each BM stromal cell component for relapse. The AUCs were calculated from the receiver operating curve of each stromal parameter for the prediction of total relapse events, early relapse (≤1 year) (n = 10), and late relapse (>1 year) (n = 4) along with the SEM. Parameters with significant predictability (AUC > 0.8) are indicated by red color. (B) Performance of various MSC subsets as predictors of relapse in AML patients. Differences in the numbers of the indicated mesenchymal subsets in the BM of CR and various relapse groups are shown in the box plot (upper panels). Predictive values of each MSC subset for early relapse (before 6 months or 1 year) or late relapse (>1 year) in comparison to CR are shown in the receiver operating curve with the AUCs and SEM. (C) Schematic illustration of leukemia-induced alterations of niches and their clinical significance.
Fig 1.

A. freshly isolated BM-MNCs (CD45/31/235a- gated cells)

B. Relative percentage (%)

C. SA-β-gal* cells (%)

D. No. CFU-F

E. No. CFU-F

**P > .05**

Normal | AML
---|---
90.9% | 52.9%
9.1% | 13.7%

Normal MSC | AML MSC
---|---
10 | 20
5 | 15

Normal (36.5yo) | AML (43.3yo)
---|---
600 | 300
200 | 100

Normal Remission (36.5yo) | (40.2yo)
---|---
150 | 100
50 | 25
Fig 2.

A

Normal CD34*cells

AML CD34*cells

Normal MSC

Sort for MSC

Gene expression changes of MSC induced by normal or AML cells

B

C

Up-regulated in MSCs with AML CD34+

AMINE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY (41)
AMINE_TRANSPORT (38)
AXON_GUIDANCE (22)
CHEMOKINE_RECEPTOR_BINDING (43)
AMINO_ACID_TRANSPORT (26)
CARBOXYLIC_ACID_TRANSMEMBRANE_TRANSPORTER_ACTIVITY (44)
CARBOXYLIC_ACID_TRANSPORT (41)
CHEMOKINE_ACTIVITY (42)
ORGANIC_ACID_TRANSPORT (42)
G_PRORTEIN_COUPLD.Receive_Receptor_BINDING (54)

Down-regulated in MSC with AML CD34+

SPINDLE (38)
CHROMOSOME (122)
CHROMATIN_BINDING (32)
CHROMOSOMAL_PART (95)
M_PHASE_OF_MITOTIC_CELL_CYCLE (84)
LYASE_ACTIVITY (69)
REPLICATION_FORK (18)
DNA_DEPENDENT_DNA_REPLICATION (50)
DNA_REPLICATION (94)
CELL_CYCLE_PHASE (166)
Fig 3.
Fig 5.
Fig 6.
Fig 7.

Table A

<table>
<thead>
<tr>
<th></th>
<th>MSC</th>
<th>EC</th>
<th>P-MSC</th>
<th>OB</th>
<th>CFU-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (se)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Relapse vs. CR (total)</td>
<td>0.78 (0.07)</td>
<td>0.63 (0.09)</td>
<td>0.72 (0.09)</td>
<td>0.70 (0.09)</td>
<td>0.69 (0.09)</td>
</tr>
<tr>
<td>Early relapse (≤1yr) vs. CR</td>
<td>0.73 (0.09)</td>
<td>0.65 (0.09)</td>
<td>0.80 (0.08)</td>
<td>0.63 (0.11)</td>
<td>0.73 (0.10)</td>
</tr>
<tr>
<td>Late relapse (&gt;1yr) vs. CR</td>
<td>0.91 (0.06)</td>
<td>0.57 (0.15)</td>
<td>0.52 (0.25)</td>
<td>0.88 (0.08)</td>
<td>0.59 (0.18)</td>
</tr>
</tbody>
</table>

Diagram B

- **Early relapse**
  - P-MSC: AUC (0.01)
  - MSC: AUC (0.01)

- **Late relapse**
  - OB: AUC (0.05)

Diagram C

- Reprogramming of BM niche by LSC
- Altered Leukemic Microenvironment
  - Leukemic dominance
  - Leukemic suppression
- Heterogeneity in stromal remodeling
- BM at initial diagnosis
  - LSC: Loss of P-MSC
  - OB & MSC
- Prognosis after treatment
  - Complete Remission (5-8yrs)
  - Early Relapse (≤6mo,1yr)
  - Late Relapse (>1yr)
Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous leukemogenesis in acute myeloid leukemia


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