Bone marrow–derived mesenchymal stromal cells promote resistance to tyrosine kinase inhibitors in chronic myeloid leukemia via the IL-7/JAK1/STAT5 pathway

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Chronic myeloid leukemia (CML) is caused by the fusion of the BCR activator of RhoGEF and GTPase activating protein (BCR) and ABL proto-oncogene, the nonreceptor tyrosine kinase (ABL) genes. Although the tyrosine kinase inhibitors (TKIs) imatinib (IM) and nilotinib (NI) have remarkable efficacy in managing CML, the malignancies in some patients become TKI-resistant. Here, we isolated bone marrow (BM)-derived mesenchymal stem cells (MSCs) from several CML patients by Ficoll-Hypaque density-gradient centrifugation for coculture with K562 and BV173 cells with or without TKIs. We used real-time quantitative PCR to assess the level of interleukin 7 (IL-7) expression in the MSCs and employed immunoblotting to monitor protein expression in the BCR/ABL, phosphatidylinositol 3-kinase (PI3K)/AKT, and JAK/STAT signaling pathways. We also used a xenograft tumor model to examine the in vivo effect of different MSCs on CML cells. MSCs from patients with IM-resistant CML protected K562 and BV173 cells against IM- or NI-induced cell death, and this protection was due to increased IL-7 secretion from the MSCs. Moreover, IL-7 levels in the BM of patients with IM-resistant CML were significantly higher than in healthy donors or IM-sensitive CML patients. IL-7 elicited IM and NI resistance via BCR/ABL-independent activation of JAK1/STAT5 signaling, but not of JAK3/STAT5 or PI3K/AKT signaling. IL-7 or JAK1 gene knockdown abrogated IL-7–mediated STAT5 phosphorylation and IM resistance in vitro and in vivo. Because high IL-7 levels in the BM mediate TKI resistance via BCR/ABL-independent activation of JAK1/STAT5 signaling, combining TKIs with IL-7/JAK1/STAT5 inhibition may have significant utility for managing CML.

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by the presence of the BCR/ABL fusion gene, which results from the translocation of the t(9,22)(q34;q11) loci (1–3). Imatinib mesylate (IM), as a molecularly-targeted therapeutic agent, has been proven to be a remarkably effective drug in the treatment of BCR/ABL-positive leukemias (4–7). Although most patients in chronic phase could achieve complete cytogenetic remissions, not all CML patients respond equally well. Over time, some CML patients become refractory to further treatment, and almost all patients have detectable levels of BCR/ABL+ cells, which indicate that IM does not eliminate minimal residual disease (8, 9). The underlying mechanisms of the existence of the residual BCR/ABL+ cells are poorly understood. Association of BCR/ABL kinase mutations with tyrosine kinase inhibitor (TKI) resistance has been frequently reported (10–14). To overcome this resistance, the second-generation ABL kinase inhibitors, such as nilotinib (NI) and dasatinib (DA), were introduced into clinical practice (15–18). However, recent studies have shown that DA and NI failed to achieve complete eradication of the disease in IM-resistant CML (19, 20). Notably, hematologic or cytogenetic response to NI was not dependent on whether kinase mutations exited in IM-resistant CML patients. These results imply that BCR/ABL-independent mechanisms may lead to TKI resistance during progression of the disease.

The bone marrow hematopoietic microenvironment is a rich source of growth factors and cytokines that may provide survival signals to the residual CML cells (21–23). Williams et al. (24) have reported that cytokines in the bone marrow microenvironment can facilitate leukemic proliferation and confer resistance to imatinib in mouse BCR/ABL+ , Arf-null lymphoblastic leukemia resistance. In this report, we were prompted to address the potential MSC-derived cytokines that are involved in resistance to BCR/ABL inhibitors in CML.

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2 The abbreviations used are: CML, chronic myeloid leukemia; TKI, tyrosine kinase inhibitor; IM, imatinib; NI, nilotinib; DA, dasatinib; IL-7, interleukin 7; MSC, mesenchymal stem cell; RMSC, IM-resistant MSC; SMSC, IM-sensitive MSC; NCMS, normal control MSC; BM, bone marrow; PI3K, phosphatidylinositol 3-kinase; KI, kinase inhibitor; qPCR, quantitative PCR; LICs, Leukemia-Initiating pre-B Cells.
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Results

RMSCs mediate apoptosis and enhance maintenance of CML cells following TKI treatment

To study the effect of different bone marrow MSCs on the survival and apoptosis of K562 and BV173 cells induced by IM or NI, bone marrow MSCs from IM-sensitive CML patients (SMSCs), IM-resistant CML patients (RMSCs), and healthy donors (NCMSCs) were isolated from bone marrow, as shown in Fig. 1. The morphology of these MSCs was similar and fibroblast-like spindle-shaped. More than 98% of the cells were negative for surface markers such as CD34, CD14, HLA-DR, and CD45. However, more than 95% of the cells possessed MSC markers, such as CD29, CD90, CD73, CD105 (Fig. 1G). Although these cells had similar surface markers, their fusion time was different. As shown in Fig. 1J, the 90% fusion time for RMSCs was significantly longer (31.5 ± 1.29) than for NCMSCs and SMSCs.

The K562 and BV173 cells were then cocultured with different MSCs. Coculture with a low concentration of bone marrow MSCs had no significant effect on the proliferation of leukemia cells, but the high concentration of bone marrow MSCs could significantly inhibit the proliferation of leukemia cells, and the inhibitory effect was enhanced with the increase of the proportion of MSCs, which showed that the proliferation inhibition was concentration-dependent (Fig. 2A). The effect of MSCs on the proliferation of leukemic cells was also time-dependent. The inhibitory effect of MSCs on the proliferation of leukemic cells was enhanced over time. There was no significant difference in the three types of bone marrow MSCs on the proliferation of leukemia cells (Fig. 2B).

However, after TKI treatment, with the increase of the concentration of IM or NI, the proliferation rate of leukemia cells gradually decreased. NCMSCs, SMSCs, and RMSCs showed the same trend of antagonizing the effects of IM or NI. However, under the same drug concentration (1 and 5 μM IM or 100 and 300 nM NI), the cell proliferation rate of the K562 + RMSCs group was significantly higher than NCMSCs and SMSCs groups (Fig. 2, C and D). Viability of K562 cells after TKI treatment in the absence or presence of different MSCs was also measured (Fig. 2, E and F). The IC_{50} values of IM and NI in the RMSC group were 7.70 and 0.35 μM, respectively. The most significantly decreased apoptotic rate was observed in the RMSC group after NI treatment (Fig. 2G).

These results suggest that factors secreted by RMSCs promoted IM/NI resistance of K562 and BV173 cells during TKI treatment by reducing apoptosis and increasing the proliferation of both cells.

RMSCs mediate BCR/ABL-independent NI resistance

Next, to assess whether RMSC-induced IM/NI resistance was BCR/ABL-dependent, we used Western blotting to detect the BCR/ABL kinase activation status. The phosphorylated BCR/ABL (pBCR/ABL), and the substrate of BCR/ABL, Crk-like protein (pCrkL), and STAT5 (pSTAT5) were evaluated. Although NI completely dephosphorylated BCR/ABL (and CrkL) in the RMSC group, STAT5 phosphorylation still existed (Fig. 3). Thus, RMSC-mediated resistance was associated with a BCR/ABL-independent activation of STAT.

IL-7 mediates TKI resistance induced by RMSCs

STAT5 signaling is normally triggered by cytokines. Upon binding to their receptors, cytokines activate members of the JAK family and STAT5. Singh et al. (25) used a naive screening approach and identified that IL-7 was the most potent leukemia-microenvironment cytokine that attenuates the response of murine Ph+ BCR/ABL.WT LICs and the human Ph+ ALL cell line SUP-B15 to BCR-ABL-Kls. Here, we measured the concentrations of IL-7 in different bone marrow and in the conditioned medium of different MSCs. The results showed IL-7 levels in the bone marrow of RCML were significantly higher than those of NC and SCML (Fig. 4A). The same result was observed in the conditioned medium of MSCs (Fig. 4B).

We also detected the expression of IL-7 mRNA in the bone marrow of CML patients by qPCR. The result indicated that IL-7 was expressed at a higher level in the IM-resistant patients than in the IM-sensitive patients (Fig. 4C).

When IL-7 antibody was added to the RMSC coculture group, the protection of RMSCs was significantly attenuated. CML cell proliferation decreased and apoptotic rate increased (Fig. 4, D and F). The same result was observed when the IL-7 gene was silenced (Fig. 4, E and G). Taken together, IL-7 plays an important role in conferring BCR/ABL-independent TKI resistance.

IL-7 protects CML cells from IM-induced apoptosis through activation of the JAK1/STAT5 pathway

We next analyzed the IL-7–associated signal transduction events that were associated with TKI resistance in CML cells. Western blotting indicated that JAK1, but not JAK3, was activated after IM treatment, especially in the RMSC group. There was a significant increase in pJAK1 and pSTAT5 proteins in K562 and BV173 cells cocultured with RMSCs (Fig. 5, A and B). Inhibition of IL-7 expression by siRNA significantly reduced protein levels of pJAK1 and pSTAT5 in both CML cells and abolished the protective effect of IL-7 against IM treatment. This effect also was observed when we used the anti-IL-7 antibody to neutralize IL-7. Silencing of the JAK1 gene decreased the level of pSTAT5 indicating that STAT5 is the main downstream target kinase involved in the resistance. When cells were treated with IL-7 antibody and STAT5 inhibitor (pimozide) simultaneously, viability of K562 cells was significantly decreased (Fig. 5D), although the results showed that the PI3K/AKT signaling pathway was not involved (Fig. 5C).

RMSCs protect CML cells from tyrosine kinase inhibitors through IL-7/JAK1/STAT5 signaling in vivo

In this study, K562 cells alone or together with different MSCs were transplanted subcutaneously into BALB/c-nu mice to observe the outgrowth of the tumor and analyze the characteristics of tumor cells (Fig. 6).

Tumor nodules were first observed at 10 days in group K562 cells alone or mixed with MSCs after injection. In the vehicle control-treated group, tumor growth was significantly enhanced in the K562 alone group compared with other groups
(Fig. 6C). After IM treatment, tumor nodules of K562 + RMSCs group increase rapidly in the IM-treated group. The biggest tumor volume of the K562 + RMSCs group achieved 401 ± 8 mm³ on day 40. Notable tumor growth inhibition was observed in K562 alone, K562 + NCMSCs, K562 + SMSCs, K562 + RMSCs, and K562 + RMSCs + IL-7 siRNA groups. Meanwhile,
Figure 2. RMSCs protect K562 and BV173 cells from TKI treatment. A, proliferation of K562/BV173 cells cocultured with different concentrations of MSCs after 24 h. B, proliferation of K562/BV173 cells cocultured with MSCs at different times. The ratio of MSCs and K562/BV173 cells was 1:1. C, proliferation of K562/BV173 cells cocultured with different MSCs after IM treatment. The ratio of MSCs and K562/BV173 cells was 1:1. After K562/BV173 cells were cocultured with MSCs for 48 h, IM was added for another 24 h. The final concentration of IM was 200 nM, 1 μM, and 5 μM. D, proliferation of K562/BV173 cells cocultured with different MSCs after NI treatment. The ratio of MSCs and K562/BV173 cells was 1:1. After K562/BV173 cells were cocultured with MSCs for 48 h, NI was added for another 24 h. The final concentration of NI was 30, 100, and 300 nM. E, viability of K562 cells after IM treatment in the absence or presence of different MSCs. F, viability of K562 cells after NI treatment in the absence or presence of different MSCs. G, representative FACS plot for annexin-V/PI-positive K562/BV173 cells cocultured with different MSCs after TKI treatment. *, p < 0.05 Student’s t test.
tumor weight of the K562 alone group was significantly higher than that of other groups without IM treatment. However, no significant differences in tumor weight were found among other groups. But after IM treatment, the mean tumor weight of K562 + RMSCs was higher than that of other groups.

In addition, the tumor tissue cells in the group injected with K562 plus RMSCs were positive for pJAK1 and pSTAT5 (Fig. 7). After knocking out the RMSc gene of IL-7, the expression levels of pJAK1 and pSTAT5 protein in tumor tissue decreased.

These results indicated that RMSCs could promote IM and NI resistance of CML cells in vivo. This xenograft study also showed that RMSCs protect CML cells from tyrosine kinase inhibitors through IL-7/JAK1/STAT5 signaling in vivo.

**Discussion**

The BCR/ABL kinase inhibitors (BCR/ABL-KIs) imatinib, nilotinib, and dasatinib have proved to be effective in many CML patients (26), however; these TKIs provide only transient anti-leukemia effects in Ph+ CML patients. This reflects disease persistence under TKI therapy. The mechanisms of TKI resistance are still poorly understood. BCR/ABL kinase mutations, P-glycoprotein, SRC family kinases, as well as TP53 deletion may also contribute to CML persistence (20, 27–30). Together, most IM resistances are in line with a concept of BCR/ABL-dependent survival.

Here, we identified that IL-7, a hematopoietic cytokine, conferred BCR/ABL-independent NI and IM resistance by activating JAK1/STAT5. CML pathogenesis is partly controlled by the complex network of cytokine- and cell contact-mediated interactions between tumor cells and the microenvironment. There is mounting evidence that microenvironmental factors can contribute to CML chemoresistance (31–34). Aberrant production of hematopoietic cytokines has long been suggested to play a role in CML biology (35). Some of these cytokines/chemokines such as GM-CSF, VEGF, IL-6, IL-15, and MIP-1a, etc., have been shown to promote the proliferation of CML cells after TKI withdrawal (36–39).

IL-7 is a hematopoietic growth factor produced by bone marrow and thymic cells, which regulates the development of human B and T cells (40–45). Some studies have documented that IL-7 plays a vital role in certain types of lymphoma and leukemia (46–49). Singh et al. (25) used a naive screening approach and identified IL-7 as the most potent leukemia-microenvironment cytokine that attenuates the response of murine Ph+ BCR/ABLWT LICs and the human Ph+ ALL cell line SUP-B15 to BCR/ABL KIs. IL-7 also confers an aggressive dasatinib-resistant phenotype to Ph+ ALL in vivo. Implantation of IL-7+/−, but not IL-7−/−, into host mice with relatively small numbers of BCR/ABLWT LICs led to decreased survival. However, IL-7− did not affect the potency of tested non-BCR/ABL–targeted drugs. Thus, IL-7 drives a specific cellular response that provides resistance to all clinically-available BCR/ABL-KIs.

We describe here a novel role for IL-7 secretion as an important mechanism for BCR/ABL+ cells to resist IM and NI in different cell systems. When BCR/ABL signaling is inhibited, CML cells can adapt to the growth factor-driven signaling provided by MSCs. We showed that the IL-7−–mediated JAK1/STAT5 but not the JAK3/STAT5 or PI3K/AKT pathway activation circumvents the need for BCR/ABL signaling to maintain survival or proliferation.

Our results demonstrate that inhibition of IL-7 or JAK1 signaling can effectively sensitize CML cells to IM and diminish the resistance to TKIs. This compensatory survival support provided by activated JAK1 may be vital to the evolution of drug resistance.

The mechanism of the abnormal secretion of IL-7 is unclear at present. In this study, we suggest that TKI therapy itself triggered the development of resistance to TKIs. It is known that expression changes are much easier to achieve than genetic mutations by tumor cells. MSCs exposed to TKIs abnormally produce prosurvival factors in response to IM selection pressure at the initial stages of the resistance. Thus, IL-7 overexpression could be an early resistance mechanism before kinase mutations emerge. Under continuous drug exposure, genetic alterations (BCR/ABL mutations) emerge ultimately at the time of IM resistance development.
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**Figure 4.** IL-7 mediates IM and NI resistance. **A**, IL-7 levels in different bone marrow samples. **B**, IL-7 levels in conditioned medium produced by different MSCs. **C**, detection of expression of IL-7 in mononuclear cells of bone marrow. **D**, proliferation of K562 cells after TKI treatment in the presence of RMSCs with or without anti-IL-7. **E**, proliferation of K562 cells after TKI treatment in the presence of RMSCs with or without siIL-7. **F**, representative FACS plot for propidium iodide-gated annexin-positive K562 cells after TKI treatment in the presence of RMSCs with or without anti-IL-7. **G**, representative FACS plot for propidium iodide-gated annexin-positive K562 cells after TKIs treatment in the presence of RMSCs with or without SiIL-7. *, \( p < 0.05 \) Student’s t test.
Because inhibition of JAK1/STAT5 blocks IL-7 signaling, JAK1/STAT5 inhibitors may overcome cytokine-dependent TKI resistance. Here, we suggest the combination of IL-7/JAK1/STAT5 signaling inhibitors with TKIs may overcome the protective effect of the microenvironment and have significant clinical implications in CML treatment.
Experimental procedures

CML patient samples and isolation of human bone marrow MSCs

16 CML patients at the Department of Hematology, The Second Affiliated Hospital of Nanchang University (Jiangxi, China), were enrolled in this study, including eight IM-sensitive CML patients (four males and four females) and eight IM-resistant CML patients (five males and three females). The median age of these CML patients was 51 years (range 37–66 years). All of them fulfilled the standard diagnostic criteria for IL-7/JAK1/STAT5 pathway in chronic myeloid leukemia.
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Table 1
Characteristics of the individuals enrolled in this study
The following abbreviations are used: ND, not done; NC, healthy donors; M, male; F, female; WBC, white blood cells.

| Patient no. | Disease status | Gender | Age, years | WBC count (× 10^9/liter) | Karyotype                  | Anti-CML therapy  |
|------------|----------------|--------|------------|--------------------------|----------------------------|-------------------|
| 1          | SCML           | F      | 42         | 65.2                     | t (9;22) (q34;q11)         | Imatinib mesylate |
| 3          | SCML           | F      | 51         | 278.9                    | t (9;22) (q34;q11)         | Imatinib mesylate |
| 4          | SCML           | M      | 37         | 23.5                     | t (9;22) (q34;q11)         | Imatinib mesylate |
| 5          | SCML           | F      | 39         | 45.3                     | Complex                    | Imatinib mesylate |
| 6          | SCML           | F      | 56         | 320.5                    | t (9;22) (q34;q11)         | Imatinib mesylate |
| 7          | SCML           | M      | 60         | 477.3                    | t (9;22) (q34;q11)         | Imatinib mesylate |
| 8          | SCML           | M      | 45         | 58.6                     | t (9;22) (q34;q11)         | Imatinib mesylate |
| 9          | RCML           | M      | 59         | 32.5                     | t (9;22) (q34;q11)         | Imatinib mesylate |
| 10         | RCML           | M      | 40         | 246.8                    | t (9;22) (q34;q11)         | Imatinib mesylate |
| 11         | RCML           | F      | 54         | 71.4                     | t (9;22) (q34;q11)         | Imatinib mesylate |
| 12         | RCML           | F      | 47         | 105.6                    | t (9;22) (q34;q11)         | Imatinib mesylate |
| 13         | RCML           | M      | 61         | 83.2                     | Complex                    | Imatinib mesylate |
| 14         | RCML           | M      | 43         | 49.3                     | t (9;22) (q34;q11)         | Imatinib mesylate |
| 15         | RCML           | M      | 65         | 309.7                    | t (9;22) (q34;q11)         | Imatinib mesylate |
| 16         | RCML           | M      | 66         | 255.4                    | t (9;22) (q34;q11)         | Imatinib mesylate |
| 17         | NC             | M      | 52         | 9.2                      | ND                         | None              |
| 18         | NC             | M      | 46         | 8.9                      | ND                         | None              |
| 19         | NC             | F      | 38         | 8.8                      | ND                         | None              |
| 20         | NC             | F      | 53         | 7.5                      | ND                         | None              |
| 21         | NC             | F      | 61         | 10.2                     | ND                         | None              |
| 22         | NC             | F      | 47         | 9.3                      | ND                         | None              |
| 23         | NC             | M      | 49         | 8.5                      | ND                         | None              |
| 24         | NC             | F      | 58         | 6.4                      | ND                         | None              |

CML. BCR-ABL1 transcript levels <10% at 3 months and <1% at 6 months after IM treatment were defined IM-sensitive CML patients, whereas BCR-ABL1 transcript levels >10% at 6 months and >1% at 12 months of defined resistance were according to the criteria proposed by the European Leukemia Net and NCCN guidelines. Eight healthy donors were included in the study as the control group (five males and three females). The median age was 51 years (range 38 – 61 years). All the individuals provided written informed consent prior to enrollment. Characteristics of the individuals enrolled in this study are shown in Table 1. This study was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Nanchang University. In the case of obtaining human bone marrow, studies abided by the Declaration of Helsinki principles.

Bone marrow samples were harvested by aspiration from IM-sensitive CML patients (SCML), IM-resistant CML patients (RCML), and healthy donors (NC) after written informed consent. The plasma samples were collected by centrifugation and stored at −80 °C. Mononuclear cells were isolated by 1.073 g/ml Ficoll-Hypaque density-gradient centrifugation, as described previously. 1–2 × 10^6 mononuclear cells were seeded in 25-cm² culture flasks (Costar, Corning, Germany) and maintained in Dulbecco’s modified Eagle’s medium/F-12 (Gibco) medium containing 10% fetal bovine serum (Gibco), 2.0 mM glutamine, penicillin (100 units/ml), and streptomycin (100 units/ml) at 37 °C in a humidified incubator with 5% CO₂ atmosphere. After 8 days, nonadherent cells were removed, and the medium was changed every 3–4 days. These cells were trypsinized (0.25% trypsin with 0.1% EDTA, Sigma) when they reached 90% confluency and were replated at a density of 8 × 10^4 cells/cm² into 25-cm² culture flasks (~1:3). MSCs from passage 3 or 4 were used for the experiments.

Immunophenotyping of MSCs was performed before experiments. The following monoclonal antibodies were used: anti-CD45-FITC; anti-CD34-FITC; anti-CD73-FITC; anti-CD29-PE; anti-CD105-PE; CD14-FITC; CD90-PE; and HLA-DR-PE (eBioscience, CA). Labeled cells were analyzed using BD FACSDiva flow cytometry (BD FACSCanto™ II, BD Biosciences).

Chronic myeloid leukemia cells lines

The human CML cell line K562 was kindly provided by Prof. Xiaozhong Wang (Nanchang University, Nanchang, China). BV173 cell were purchased from Shanghai Fanyan Biotechnology Co., Ltd., and K562 and BV173 cells were authenticated by STR DNA fingerprinting.

Both cells were cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 units/ml streptomycin, 100 units/ml penicillin, and 2.0 mM glutamine (Gibco) at 37 °C in a humidified incubator with 5% CO₂ atmosphere.

Drugs and reagents

IM and NI were obtained from Sigma-Aldrich (Hamburg, Germany). Stock solutions of 10 mM were prepared by dissolving in dimethyl sulfoxide (DMSO) and stored in aliquots at −20 °C. Fresh working solutions were prepared in RPMI 1640 medium for each experiment. The neutralizing antibodies to IL-7 were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). Pimozide, the inhibitor of STAT5, were purchased from Sigma-Aldrich (Hamburg, Germany).

Figure 7. Increased expressions of pJAK1 and pSTAT5 were confirmed by immunofluorescence staining and Western blotting in tumor tissue. A, expression of pJAK1 and pSTAT5 in tumor tissue by immunofluorescence staining. B, representative blots and densitometry analysis of JAK1, pJAK1, STAT5, pSTAT5, and β-ACTIN in tumor tissue. DAPI, 4',6-diamidino-2-phenylindole.
Cell proliferation assay

NCMCSs, SMSCs, and RMSCs were cocultured with K562/BV173 cells in different ratios at different time points. $1 \times 10^5$ MSCs/well were seeded on 96-well plates. The ratios of MSCs to leukemic cells were 1:10, 1:5, 1:1, and 5:1. They were placed in a 5% CO$_2$ humidified atmosphere at 37 °C for 24, 48, and 72 h. After 48 h, IM was added to the cultures for a further 24 h at 200 cells/well. Then K562 or BV173 cells in cocultures were isolated by careful pipetting with ice-cold PBS, repeated twice. Cell proliferation was assessed by using Cell Counting Kit 8 (CCK8 kit, Dojindo, Shanghai, China) as described previously. The absorbance of each well at 450 nm was measured using a microplate ELISA reader (model FL 311, Bio-Tek Instruments, Winooski, VT).

Apoptosis analysis measurement

K562 cells were cocultured with NCMCSs, SMSCs, and RMSCs at a ratio of 1:1 for 48 h, and then IM or NI was added to the cultures for a further 24 h. Apoptosis of K562 cells was measured by using the annexin-V/propidium iodide apoptosis detection kit (BD Biosciences) according to the manufacturer’s recommendations. $5 \times 10^5$ cells were washed twice with ice-cold PBS and then resuspended in 100 μl of binding buffer. Cells were stained for 30 min with 5 μl of FITC-conjugated anti-annexin V antibody and 10 μl of propidium iodide. The cells were analyzed using BD FACSDiva flow cytometry (BD FACSCanto™ II). The percentage of both early apoptotic and late apoptotic cells was quantified.

Western blot analysis

The nuclear extracts and total proteins were prepared from treated K562 and BV173 cells, as described previously. Five groups of CML cells were lysed in lysis buffer. Protein concentrations were measured, and 20 μg of total protein from each condition was fractionated on 10% SDS-polyacrylamide gel at 100 V for 2 h, and then the protein was transferred to 0.2-mm polyvinylidene difluoride membranes. Membranes were blocked by incubation in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% milk at room temperature for 1 h and incubated with specific antibodies against JAK1 (3331, Cell Signaling Technology), phospho-JAK1 (Tyr-1022/1023) (3331, Cell Signaling Technology), JAK3 (8827, Cell Signaling Technology), phospho-JAK3 (Tyr-980/981) (5031, Cell Signaling Technology), STAT5 (9363, Cell Signaling Technology), phospho-STAT5 (Tyr-694) (4322, Cell Signaling Technology), c-Abl (2862, Cell Signaling Technology), pCrkL (Proteintech), and phospho-JAK3 (Tyr-980/981). After washing, the membrane was incubated with horseradish peroxidase–conjugated secondary antibodies and then visualized with enhanced chemiluminescence.

Enzyme linked immunosorbent assays (ELISA)

IL-7 concentration in the conditioned media from different MSCs was measured using the human IL-7 high-sensitivity ELISA kit (Abcam, Cambridge, UK). Conditioned culture media were collected and centrifuged at 1000 rpm for 5 min and then stored at −80 °C until measurement. The detection range was 1.6–100 pg/ml, and the sensitivity was less than 0.19 pg/ml. The protocol was performed according to the manufacturer’s instructions. The absorbance was measured on an ELISA Reader (Molecular Devices, Ismaning, Germany) at 450 nm.

Real-time qPCR

To assess the pattern of IL-7 expression between MSCs from different CML patients, total RNA in mononuclear cells was isolated by using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. One microgram of total RNA was reverse-transcribed in a 20-μl reaction volume containing 4 μg of total RNA and SuperScript II RT according to the instructions of the manufacturer (Takara).

Real-time quantitative PCR was performed by using SYBR Green I in 20 μl of reaction mixture. The amplification thermal profile included initial denaturation at 95 °C for 30 s, followed by 40 cycles of amplification (95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s). The expression of the housekeeping gene β-actin was used to normalize the IL-7 expression. The following primers were designed: IL-7 forward, 5-GTGAC-TATGGGCGTGTAGAG-3; IL-7 reverse, 5-GCTACT-GGCAACAGAAACGG-3; β-actin forward, 5-ACTCTT-GAGGCTCTTCTCTC-3; β-actin reverse, 5-CTAGGATCTCTCTTCAGATCT-3. All assays were performed in triplicate on an ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA). Normalized expression values were calculated by using the formula: $2^{-\Delta\Delta C_T}$.

siRNA transfections

For RNAi studies, siRNAs directed against IL-7 and JAK1 were purchased from Genepharma Co. (Shanghai, China). Transfection was performed using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocol. Knockdown efficiency was determined by Western blotting. Cells were harvested after 48 h for downstream experiments.

Xenograft tumor model

To evaluate the in vivo effect of different MSCs on CML cells, female BALB/c-nu mice were purchased from the Laboratory Animal Center of Academy of Sciences (Hunan, China) and were bred under specific pathogen-free conditions. The mice were used for experiments at 6–8 weeks of age. Mice were allowed to acclimate for 1 week after arrival. K562 cells admixed with or without different MSCs were injected subcutaneously for evaluation of tumor formation. The 50 BALB/c-nu mice were randomly divided into five groups ($n = 10$). All groups received a subcutaneous injection of 200 μl of PBS per mouse into the front of the right backside of mice containing the following: 1) $1 \times 10^7$ K562 cells; 2) $1 \times 10^7$ K562 cells mixed with $1 \times 10^7$ NCMSCs; 3) $1 \times 10^7$ K562 cells mixed with $1 \times 10^7$ SMSCs; 4) $1 \times 10^7$ K562 cells mixed with $1 \times 10^7$ RMSCs; 5) $1 \times 10^7$ K562 cells mixed with $1 \times 10^7$ siRMSCs (RMSCs in which the IL-7 gene was knocked down). Animals were monitored daily for changes in weight, tumor size, side effects of treatment, and signs of any sickness. Tumor growth was evaluated by measuring the length and width of the tumor mass using a caliper. The treated groups were given imatinib (100 mg/kg), adminis-
tered by oral gavage daily for 30 days. The control group was treated with vehicle. Tumor volumes were calculated by using the formula: \( V = \frac{1}{2} \times (\text{length} \times \text{width}^2) \). All the mice were sacrificed 40 days after tumor inoculation by cervical dislocation. Tumors were resected, measured, and photographed, and the tumor tissues were analyzed by immunofluorescence. Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body. The experimental procedures were approved by the Ethics Committee of The Second Affiliated Hospital of Nanchang University.

**Immunofluorescence staining**

Tumor samples were fixed in 4% formaldehyde solution for several days and embedded in paraffin. Then tumor samples were sectioned and mounted on slides. The slices were rehydrated through a gradient of alcohol and incubated with the following primary antibodies: rabbit antibodies against human, phospho-JAK1 (Tyr-1022/1023) (3331, Cell Signaling Technology) and phospho-STAT5 (Tyr-694) (4322, Cell Signaling Technology), respectively, overnight at 4°C. They were also stained with 4,6-diamidino-2-phenylindole to visualize the nuclei. Immunofluorescence was examined by fluorescence microscopy. Data were analyzed by Image-pro Plus version 6.0 software.

**Statistical analysis**

All data are presented as mean ± S.D., using the SPSS system package for statistical analysis. Paired data were analyzed using the paired Student's t test. One-way analysis of variance was used for multiple groups of data. A \( p \) value < 0.05 was considered significant.

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