An interaction between hepatocyte growth factor and its receptor (c-MET) prolongs the survival of chronic lymphocytic leukemic cells through STAT3 phosphorylation: a potential role of mesenchymal cells in the disease

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
Chronic lymphocytic leukemia cells are characterized by an apparent longevity *in vivo* which is lost when they are cultured *in vitro*. Cellular interactions and factors provided by the microenvironment appear essential to cell survival and may protect leukemic cells from the cytotoxicity of conventional therapies. Understanding the cross-talk between leukemic cells and stroma is of interest for identifying signals supporting disease progression and for developing novel therapeutic strategies.

Design and Methods
Different cell types, sharing a common mesenchymal origin and representative of various bone marrow components, were used to challenge the viability of leukemic cells in co-cultures and in contact-free culture systems. Using a bioinformatic approach we searched for genes shared by lineages prolonging leukemic cell survival and further analyzed their biological role in signal transduction experiments.

Results
Human bone marrow stromal cells, fibroblasts, trabecular bone-derived cells and an osteoblast-like cell line strongly enhanced survival of leukemic cells, while endothelial cells and chondrocytes did not. Gene expression profile analysis indicated two soluble factors, hepatocyte growth factor and CXCL12, as potentially involved. We demonstrated that hepatocyte growth factor and CXCL12 are produced only by mesenchymal lineages that sustain the survival of leukemic cells. Indeed chronic lymphocytic leukemic cells express a functional hepatocyte growth factor receptor (c-MET) and hepatocyte growth factor enhanced the viability of these cells through STAT3 phosphorylation, which was blocked by a c-MET tyrosine kinase inhibitor. The role of hepatocyte growth factor was confirmed by its short interfering RNA-mediated knock-down in mesenchymal cells.

Conclusions
The finding that hepatocyte growth factor prolongs the survival of chronic lymphocytic leukemic cells is novel and we suggest that the interaction between hepatocyte growth factor-producing mesenchymal and neoplastic cells contributes to maintenance of the leukemic clone.

Key words: chronic lymphocytic leukemia, mesenchymal stem cells, growth factors, survival.

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Introduction

B-cell chronic lymphocytic leukemia (CLL) is characterized by the relentless accumulation of neoplastic B cells in the blood, secondary lymphoid tissue and bone marrow.[1] Despite their apparent longevity, CLL cells undergo spontaneous apoptosis *in vitro*, suggesting that interactions with the microenvironment play an essential role in their growth and survival.[2,3] In CLL patients, the extent of bone marrow infiltration by leukemic cells correlates with clinical stage and prognosis.[4] In addition, bone marrow is a preferential site of minimal residual disease and relapse. *In vitro* studies have demonstrated that the interaction between bone marrow stromal cells (BMSC) and CLL via β1 and β2 integrins rescues CLL cells from apoptosis.[3] Moreover, nurse-like cells, a particular type of stromal cells present *in vivo* in spleen and lymphoid tissues of patients,[5] and which differentiate *in vitro* from blood monocytes, protect leukemic B cells from apoptosis.[6] Within the stromal compartment, BMSC, a subset of self-renewing and multipotent cells, differentiate along several lineages, giving rise to osteocytes, chondrocytes, adipocytes, smooth muscle, endothelial and reticular cells. Stromal progenitors also form niches and through cell-to-cell contacts, cytokines and growth factors may support survival, growth and differentiation of the lympho-hematopoietic system.[7,8] In addition, BMSC display peculiar immunoregulatory activities: *in vivo*, interactions between BMSC and cells of the immune system induce anergy and modulate the functional activities of the cells.[9] BMSC inhibit T-cell responses activated by mitogens and alloantigens,[10] stimulate suppressor T cells[11] and inhibit proliferation of natural killer cells in response to interleukin-2.[12] Current data on the effects of BMSC on B cells are more limited. BMSC exert a negative control on B lymphopoiesis[13] and increase viability of normal B cells[14] while inhibiting their proliferation.[15] Here we evaluated whether bone marrow stromal components or their terminally differentiated counterparts could differently affect survival and/or expansion of leukemic B cells. We investigated whether human endothelial cells (HUVEC), BMSC or BMSC-derived cells, such as fibroblasts (HF), trabecular bone cells (TBMC), chondrocytes (HAC) or an osteoblast-like cell line (MG63) modulate neoplastic B-cell survival. Furthermore, exploiting a bioinformatic approach, we searched for genes shared among those cell lineages that supported and enhanced viability of leukemic B cells in co-culture assays, including genes for two soluble factors, CXCL12 (SDF-1α) and hepatocyte growth factor (HGF), as likely candidates. Indeed CXCL12 mediates CLL survival and migration.[16,17] However the finding that HGF may enhance CLL viability appears novel. The HGF/MET pathway is implicated in follicular dendritic - B-cell interactions[18] and HGF reduces apoptosis in multiple myeloma.[19] Moreover, HGF concentrations are higher in sera from CLL patients than in sera from controls.[20] We, therefore, assessed HGF and CXCL12 production by mesodermal lineages and analyzed HGF receptor (c-MET) expression on leukemic B cells as well as signals induced by the HGF-c-MET interaction.

Design and Methods

Patients

This study was approved by the review board of the University of Genoa, Italy. Blood samples were taken, after informed consent, from 33 untreated CLL patients: prognostic parameters are summarized in Figure 1 and detailed in *Online Supplementary Table S1*.

Purification of chronic lymphocytic leukemia cells

Peripheral blood mononuclear cells were isolated from heparinized blood samples of 33 CLL patients by density gradient centrifugation (Ficoll, Biochrom, Berlin, Germany) and subjected to immunophenotypic characterization When residual non-B cells exceeded 10%, the $CD19^+CD5^+$ population was enriched by negative selection as previously described.[21]

Immunofluorescence analysis of cell surface antigen expression on chronic lymphocytic leukemia cells

Double-color fluorescence was performed by staining 10^5 CLL cells/sample at 4°C, for 30 min with FITC-labeled anti-CD19,-CD5,-CD23 and PE-labeled anti-CD19,-CD38 monoclonal antibodies (Immunotools, Friesoythe, Germany) or isotype-matched immunoglobulins. Aliquots of 10^5 cells were also stained with anti-human-CXCR4 monoclonal antibody (R&D system, Minneapolis, MN, USA) or, after prior permeabilization, with rabbit anti-human-c-MET antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and further processed for flow cytometry as described in the *Online Supplementary Appendix*.

Primary cell cultures and cell lines used

Human BMSC were obtained from iliac crest marrow aspirates of healthy donors or CLL patients, after informed consent. Unless otherwise specified, BMSC are of allogeneic origin. Bone marrow samples were processed as previously described.[8,22] More than 85% of the BMSC were $CD49a^+$, $CD63^+$, $CD90^+$, $CD105^+$, $CD166^+$; 32% were $CD140b^+$ and less than 22% were $CD106^+$. In accordance with other studies, less than 5% of the cells were $CD146^+$.[23] Other cell types used were HAC,[24] HF, MG63 osteoblast-like cells, TBMC and HUVEC. Details of the primary cultures are given in the *Online Supplementary Appendix*. Pools from primary cultures were used to avoid bias from single donors.

Co-cultures of chronic lymphocytic leukemia and mesenchymal cells

CLL cells (5×10^5) were cultured with 2×10^3 BMSC, HF, TBMC, HAC or HUVEC or with 10^3 MG63 cells in 24-well plates with 1 mL of RPMI 1640 medium with 10% fetal bovine serum. In selected experiments a c-MET tyrosine kinase inhibitor SU11274 (10 μM, D.B.A. Italia srl, Milan, Italy, cat N. 448101), a neutralizing anti-HGF monoclonal antibody (10 μg/mL; R&D system) or anti-STAT-3 inhibitor cpd188 (5 μM; Merck Chemicals Ltd. Nottingham, UK) was added. CLL cell viability was determined by annexin V/propidium iodide staining (Bender MedSystems, Vienna, Austria) and analyzed by flow cytometry. Additional experiments with mesenchymal cells were preliminarily performed in transwell plates and subsequently by culturing 5×10^5 CLL cells in 24-well plates in complete medium supplemented with culture medium (cm: 300 μL in a final volume of 1 mL) from each different cell culture (BMSC, HF, HAC, HUVEC, MG63). ^3H-thymidine incorporation and MTT (methylthiazoletetrazolium, Sigma) assays[14,25] were also performed to evaluate any CLL proliferation.

Gene chip microarray analysis

In order to find a common expression pattern of soluble factors among cell types sustaining CLL we performed data-mining of CEL files of GeneChip Human Genome U133 Plus2 arrays (Affymetrix, Santa Clara, CA, USA) and obtained genomic data

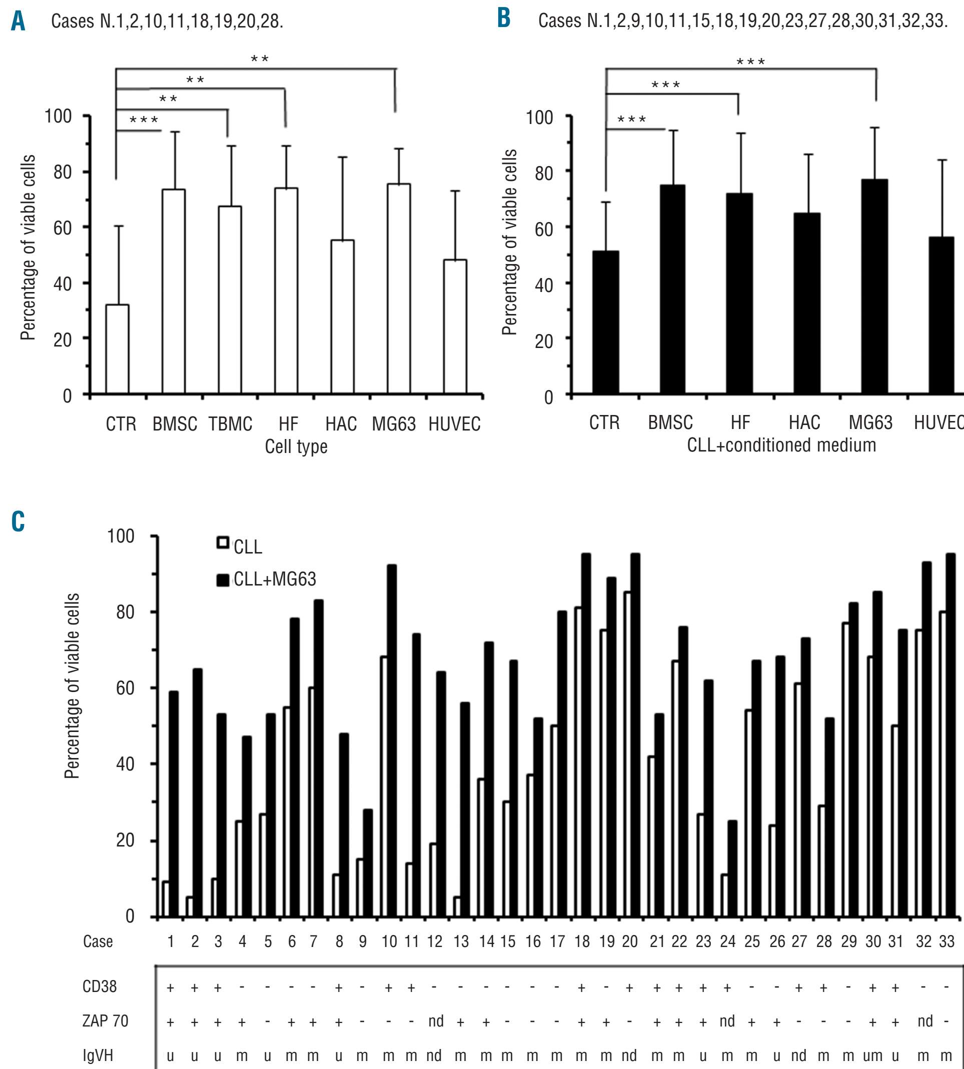

Figure 1. CLL cell viability after co-culturing with different cell types of mesenchymal origin. (A) CLL cells were rescued from apoptosis after 7 days co-culture with human BMSC, TBMC, HF, MG63. *=P<0.05; **=P<0.01; ***=P<0.001 n=8 except for MG63 n=33; (B) Increase of CLL cell survival by the addition of conditioned medium from BMSC, HF, HAC, MG63 or HUVEC; n=16. (C) CLL cells from 33 patients were rescued from apoptosis after 7-days co-cultures with MG63. Patients' prognostic markers are indicated. CD38$^+$: >30%; ZAP70$^+$: >25%; IgVH um: unmutated, m: mutated, >2%; nd: not determined.

from mRNA of BMSC from three healthy donors. Additional details are provided in the *Online Supplementary Appendix* and in *Online Supplementary Tables S2 and S3*. Sample normalization was performed among HUVEC and MG63, HAC and HF, HAC and BMSC to compare ontogenetically unrelated cells, terminally differentiated cells with a common mesodermal origin, and differentiated and progenitor cells of mesodermal origin, respectively. Consideration was limited to genes that showed at least a 4-fold expression change simultaneously on HUVEC *versus* MG63, on HAC *versus* HF and on HAC *versus* BMSC.

Evaluation of the viability of chronic lymphocytic leukemia cells after the addition of CXCL12 or hepatocyte growth factor

CLL cells (5×10^5) were challenged with human recombinant CXCL12 or HGF protein (100 ng/mL; PeproTech EC, London, UK) in 24-well plates of 1 mL volume. Where indicated SU11274 or anti-HGF monoclonal antibody was pre-incubated for 30 min with CLL prior to the addition of HGF. Viability was determined by annexin V/PI staining.

Quantification of CXCL12 and hepatocyte growth factor in spent medium

CXCL12 and HGF production was examined by enzyme-linked immunosorbent assay (ELISA) kits (Quantikine Sandwich ELISA kits; R&D) according to manufacturer's instructions. Supernatants from 48 h serum-starved cultures of BMSC, HF, HAC, HUVEC, MG63 (5×10^4 cells/well) or CLL cells (10^6 cells/well) were collected, centrifuged at 600xg to remove cellular debris and sterile-filtered. Sera from CLL patients were also collected and used.

Transcript expression for c-MET and hepatocyte growth factor in chronic lymphocytic leukemia cells

Cell aliquots from each suspension or monolayer culture were used to extract total mRNA. The PerfectPure RNA Cultured Cell Kit (5-Prime GmbH, Hamburg, Germany) and the SuperScript™ III First-strand synthesis system for reverse transcriptase polymerase chain reaction (RT-PCR) (Invitrogen) were used as indicated by the manufacturer to perform standard RT-PCR reactions. Primer sets for each gene (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*; hepatocyte growth factor, *HGF*; HGF activator, *HGFA*; and HGF receptor, *c-MET*) were derived from published sequences,[18,24,26] or specifically designed (human c-MET forward: 5'-ATACGGTCCTATGGCTGGT, reverse: 5'-TTCATAGACAATGGGATCTTC). The relative expression of c-MET was also assessed by syber-green real-time quantitative RT-PCR, as previously described.[18] Further details on qualitative and quanti-

tative RT-PCR reactions are given in the *Online Supplementary Appendix*.

Short-interfering RNA-mediated knock-down of hepatocyte growth factor in MG63 cells

All short-interfering RNA (siRNA), whether HGF-specific [cat.n. s6529 (siRNA1) and s6530 (siRNA2)] or *Silencer* select negative control unrelated siRNA (siRNACN) were from Applied Biosystems (Monza, MI, Italy),[27] and used at a final concentration of 50 nM. Transfections were performed as detailed in the *Online Supplementary Appendix*. After 24 h, CLL cells (5×10^5 cells/well) were added to transfected and mock-transfected cells, and CLL survival was assayed 48 h later by annexin-V/PI. Twenty-four hours after MG63 transfection, the HGF transcript levels were assayed by real-time RT-PCR as described above, using a specifically designed oligonucleotide pair (forward: CAATTTAGAC-CATCCCGTAATAT; reverse: CTTTCAAGTCTCGA-GAAGGGA). HGF levels were also counterchecked by ELISA, in culture media from siRNA-silenced MG63 cells 24-48 h post-transfection, as previously described.

Determination of phosphorylation of signaling pathways by western blot or intracytoplasmic staining

To evaluate STAT3, ERK1/2 and AKT phosphorylation, CLL cells (5×10^6) were incubated for 10-20 min with or without recombinant human HGF or CXCL12 (100 ng/mL). CLL cells were also co-cultured with BMSC, HF, MG63, HUVEC or their conditioned media for 24 h. A c-MET inhibitor and anti-HGF monoclonal antibody were further used in some experiments to evaluate inhibition of STAT3 phosphorylation, as previously described.[25] Membranes were probed with specific antibodies against ERK1/2, phospho-ERK1/2, Akt and phospho-Akt^{Ser473} (Cell Signaling Technology Inc., Danvers, MA, USA) or anti p-STAT3^{Tyr705} (Santa Cruz Biotechnology) and the immunocomplexes detected by an ECL system (GE Healthcare, Milan, Italy). Densitometric analysis of immunopositive bands was performed with a Gel-Doc instrument using QuantitiveOne software (Bio-Rad laboratories, Milan, Italy) and the data for each patient, collected 10-20 min after HGF addition, were normalized to the pSTAT3 band densitometric intensity of the patients' own untreated cells. An anti-pSTAT3 PE-labeled antibody (Santa Cruz Biotechnology) was utilized to detect p-STAT3^{Tyr705} by flow cytometry. After starving CLL cells in serum-free culture medium for 1 h, the cells were pre-incubated with SU11274 for 30 min where indicated and further treated with HGF (100 ng/mL) or conditioned media from BMSC or MG63 for 40 min. The cells were fixed, permeabilized, stained with the labeled antibody or an isotype control and analyzed by flow cytometry.

Statistical analysis

When appropriate two-tailed and one-tailed paired Student's t tests and Fisher's exact tests were applied. Differences were accepted as significant when the *P* value was less than 0.05 (*), less than 0.01 (**) or less than 0.001 (***).

Results

Mesoderm-derived cells support survival of neoplastic B cells in chronic lymphocytic leukemia differently

Leukemic B cells from CLL patients were co-cultured with BMSC, HF, HAC, TBMC, HUVEC or MG63 cells to investigate whether cell viability was supported by mesenchymal stromal progenitors and by terminally differentiated cells of mesodermal origin. Double staining with annexin-V/PI, showed that spontaneous apoptosis of CLL cells was significantly reduced by 7 days of co-culture with BMSC, HF, TBMC or MG63. In contrast, HUVEC or HAC did not significantly enhance the survival of CLL cells (Figure 1A). To determine the potential involvement of soluble factors we performed co-culture experiments in transwell plates. BMSC, HF and MG63 enhanced leukemic B-cell viability, even though to a lesser extent than after direct co-cultures (*data not shown*), suggesting that one or more soluble factors released are responsible for the prolonged survival observed. These results were further statistically confirmed when CLL cells were cultured with spent medium from BMSC, HF and MG63, but not that from HUVEC or HAC (Figure 1B). Based on the observation that MG63 induced a maximal effect on survival, the index of protection by MG63 cells was further analyzed in an extended number of CLL patients (n=33). MG63 significantly prolonged the viability of CLL cells in all the cases studied with a certain degree of heterogeneity [mean percentage of survival (±SD): control=43.3±26.6 *versus* 68.4±18.3 with MG63; $P<0.001$, n=33, Figure 1C]. A higher fold increase in survival (>2) was observed when leukemic cells from CLL cases with an unfavorable prognostic marker (unmutated IgVH) were co-cultured with MG63 cells (Figure 1C). This finding was statistically significant (Fisher's exact test: $P<0.02$) although the data were derived from a relatively small cohort of patients (n=33) and further investigations are needed.

Computational analysis of gene expression profiles

We used a computational approach to analyze and compare gene expression profiles in cell lineages with different effects on the survival of leukemic CLL cells to discriminate between factors produced by HF, BMSC and MG63 but not by HUVEC or HAC. Since differentiated HAC are of mesenchymal origin, although not present in the bone marrow, their expression profile was utilized as a background for those obtained from the other cell types used. A comparison of gene expression profiles between the two groups of cells supporting (BMSC, HF, MG63) or not supporting (HUVEC, HAC) CLL cell-survival yielded a list of 16 genes up-regulated in BMSC, HF and MG63 cells (*Online Supplementary Table S3*). Following a literature search only two of these 16 genes appeared related to neoplastic B-cell growth or survival: *CXCL12* and *HGF*. While it has already been reported that CXCL12 increases CLL cell-survival *in vitro*,[6] the effect of HGF on CLL had not previously been explored.

CXCR4 and c-MET, receptors for CXCL12 and for hepatocyte growth factor, respectively, are expressed on chronic lymphocytic leukemia cells

We confirmed high CXCR4 expression on CLL cells by cytofluorimetric analysis (Figure 2A, upper graphs), as reported.[28] We also determined *c-MET* mRNA expression in CLL cells by RT-PCR (Figure 2B) and by quantitative real-time RT-PCR (Figure 2C). The *c-MET* transcript was expressed in all the tested cases, while *HGF* mRNA was barely detectable in three out of six CLL cases analyzed (Figure 2B) suggesting that an autocrine loop is unlikely. Flow cytometry analysis confirmed, on purified $CD19^+$ cells, high levels of c-MET expression in almost all the cases studied (mean % ± S.D.= 69.93±0.22, Figure 2A and *Online Supplementary Table S1*).

Hepatocyte growth factor mRNA expression on different cells of mesenchymal origin

HGF mRNA expression was further analyzed in the different mesenchyme-derived cells utilized in co-cultures. With the exception of HUVEC, we detected *HGF* mRNA in all the tested cells (BMSC, HF, HAC and MG63), although to different extents, while *c-MET* mRNA was present in all the samples analyzed. Furthermore only the cell types producing high HGF levels also faintly expressed HGF activator (*HGFA*) mRNA (Figure 2D), a serine protease that cleaves and converts single chain HGF to the heterodimeric active form.[18] Lack of *HGF* and *HGFA* mRNA expression in HUVEC, and of *HGFA* in HAC, might be related to lack of protection of CLL cell survival by these mesenchymal cells.

CXCL12 and hepatocyte growth factor production by different cells of mesenchymal origin

CXCL12 was produced at high concentrations by HF (7528 pg/mL) and by BMSC from both healthy (BMSC) or leukemic donors (BMSC-CLL) (2158 and 3936 pg/mL, respectively), and in lower amounts (438 pg/mL) by MG63. In contrast, CXCL12 was absent from HUVEC and HAC culture medium (Figure 3A). HGF was produced at high levels by MG63 (9066 pg/mL), BMSC-CLL (4993 pg/mL) and BMSC (3913 pg/mL) and in a low amount by HF. HGF was, however, absent from HUVEC and HAC culture medium (Figure 3A), although a faint signal was detected in *HAC* mRNA (Figure 2D). JJN-3, a multiple myeloma cell line represents a positive control for HGF (Figure 3A). Culture medium from leukemic B cells were negative for both CXCL12 and HGF (Figure 3A), while moderate amounts of HGF were present in sera from two patients (mean: 271 pg/mL).

CXCL12 and hepatocyte growth factor overcome chronic lymphocytic leukemia cell apoptosis

Survival of CLL cells was increased by the addition of recombinant human CXCL12 (100 ng/mL) after 4 days and to an even greater extent after 10 days (Figure 3B). Recombinant HGF also enhanced cell viability, with a more evident effect after 7 days that persisted up to 14 days (Figure 3C and *Online Supplementary Table S4*). The protective effect of HGF was counteracted by addition of a c-MET inhibitor (SU11274) or an anti-HGF neutralizing monoclonal antibody (Figure 3D). In line with this result the enhanced survival of leukemic cells, induced by co-culture with mesenchymal cells that produced large amounts of HGF (BMSC and MG63), was also significantly reduced in the presence of SU11274 or the anti-HGF monoclonal antibody (Figure 3D) thus suggesting a key role of the HGF/c-MET pathway. Moreover the survival effect induced by co-culturing MG63 with CLL cells (n=3) was significantly antagonized when HGF was silenced by siRNA transfection in the osteoblast-like cell line (Figure 3E). Efficient HGF silencing in MG63 was confirmed by real-time PCR analysis of the transcript level and by ELISA determination of the protein concentration in spent culture medium (Figure 3F-3G).

Analysis of STAT3 phosphorylation in chronic lymphocytic leukemia cells

We further analyzed phosphorylation of STAT3, one of the downstream signaling molecules directly activated by HGF, after culturing leukemic B cells with BMSC, HF or MG63 cells, or after addition of HGF. STAT3 was rapidly phosphorylated in Tyr[705] (10-20 min) after HGF administration to CLL cells, as shown in representative cases in Figure 4A and in *Online Supplementary Figure S1A*. When pSTAT3 band intensities were quantified by densitometric analysis, the difference in the HGF-treated samples was statistically significant (Figure 4B, n=11). STAT3 was also activated after co-culture with MG63 and BMSC (Figure 4C). When the c-MET inhibitor SU11274 was pre-incubated with CLL cells prior to co-culture with MG63 or BMSC the levels of STAT3 phosphorylation were reduced (Figure 4D). Tyrosine kinase c-MET inhibitor also proved successful according to flow cytometry intracellular determination of pSTAT3 when cells were stimulated with recombinant HGF (Figure 4E). In fact pSTAT3 staining ranged from 13 to 23 arbitrary units (au) of mean fluorescence intensity (mfi) after HGF treatment, and was reduced to the 6-15 range after addition of the inhibitor (control values, 7-15; Figure 4E). Moreover, as evaluated in time-course experiments, Tyr[705] STAT3 was transiently phosphorylated peaking between 20-60 min and decreasing thereafter, as assessed in four different cases (*Online Supplementary Figure S2*). Conditioned medium from BMSC or MG63 cells also induced pSTAT3 phosphorylation (c.m. BMSC: 16-25 au; c.m. MG63: 41-42 au), counteracted by SU11274 pre-treatment (c.m. BMSC+ SU11274: 8-15 au; c.m. MG63+ SU11274: 8-18 au, Figure 4F). Furthermore the survival effect induced by MG63 on CLL cells was reduced when the STAT3 inhibitor cpd188[29] was added in co-cultures (Figure 4G). Similar effects were observed when leukemic cells were stimulated by exogenous HGF for 7 or 14 days. (Figure 4H). Altogether these data relate STAT3 phosphorylation to the HGF/c-MET pathway.

As already described[30] we also observed that the addition of exogenous recombinant CXCL12 induced ERK1/2 phosphorylation and a weaker signal was detected after culture with medium from BMSC but not with MG63 (*Online Supplementary Figure S1B*). After HGF treatment (10 or 20 min), or co-culture with BMSC or MG63, also AKT was phosphorylated (*Online Supplementary Figure S1C*).

Discussion

Specific mesenchymal cell populations contribute to the specialized microenvironments or niches that regulate stem cells. In CLL, bone marrow is regarded as the site of minimal residual disease suggesting that leukemic B cells may access marrow niches normally restricted to progenitor cells. *In vitro* apoptosis of CLL cells is significantly delayed by co-culture with BMSC,[3] follicular dendritic cells,[31] or nurse-like cells[6] but the mechanisms regulating CLL-mesenchymal stromal cell interactions are relatively unknown. We demonstrated that human BMSC, HF, TBMC and an osteoblast-like cell line, but not chondrocytes or endothelial cells, could significantly enhance survival. Moreover, viability was uncoupled from proliferation (*data not shown*). We further proved that a novel factor, HGF, along with the already known CXCL12, increased CLL cell viability. Interestingly we found that c-MET, the HGF receptor, is expressed in CLL cells, in addition to the already reported CXCR4,[16,28] the CXCL12 receptor. A role for CXCL12 in prolonging CLL survival has been already described in several studies[30] and the CXCL12-CXCR4 interaction has a crucial role in migration and recircula-

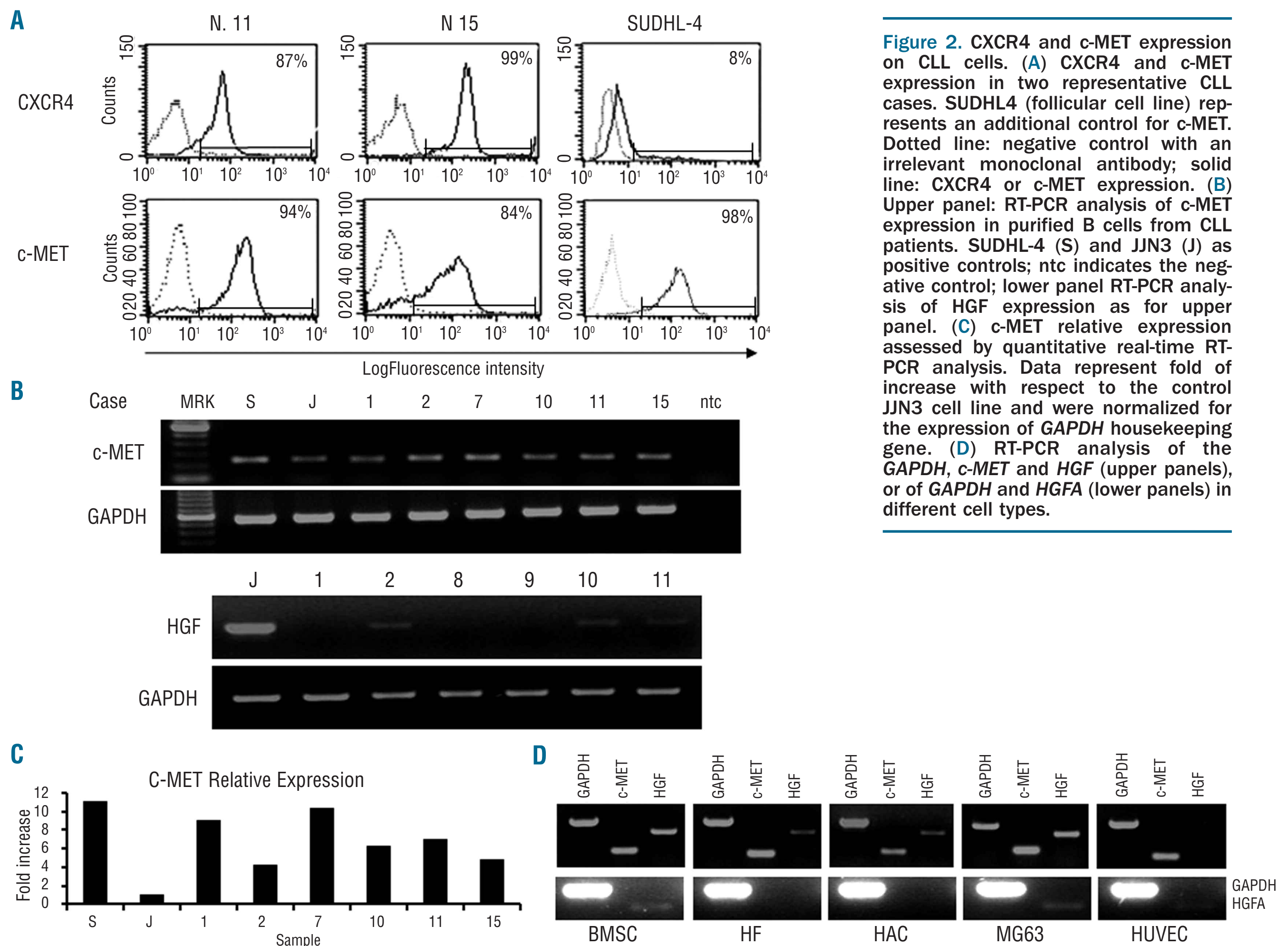

Figure 2. CXCR4 and c-MET expression on CLL cells. (A) CXCR4 and c-MET expression in two representative CLL cases. SUDHL4 (follicular cell line) represents an additional control for c-MET. Dotted line: negative control with an irrelevant monoclonal antibody; solid line: CXCR4 or c-MET expression. (B) Upper panel: RT-PCR analysis of c-MET expression in purified B cells from CLL patients. SUDHL-4 (S) and JJN3 (J) as positive controls; ntc indicates the negative control; lower panel RT-PCR analysis of HGF expression as for upper panel. (C) c-MET relative expression assessed by quantitative real-time RT-PCR analysis. Data represent fold of increase with respect to the control JJN3 cell line and were normalized for the expression of *GAPDH* housekeeping gene. (D) RT-PCR analysis of the *GAPDH*, *c-MET* and *HGF* (upper panels), or of *GAPDH* and *HGFA* (lower panels) in different cell types.

tion.[17] We demonstrated that only bone marrow mesenchymal cells producing CXCL12 (BMSC, HF, MG63) increased CLL viability in co-cultures and that survival effects induced by BMSC were blocked by an anti-CXCR4 monoclonal antibody (*data not shown*), possibly supporting the use of CXCR4 antagonists to disrupt tumor-stroma interactions in leukemia.[32] The novelty of our findings, however, resides in the identification of HGF as contributing to survival of CLL cells within bone marrow. HGF is a multifunctional cytokine whose activities, apart from supporting hepatocyte growth, include stimulation of epithelial cell motility, invasiveness and the induction of angiogenesis.[33] The receptor for HGF is c-MET, a heterodimeric surface receptor containing the tyrosine kinase domain in its transmembrane portion.[34] The HGF/c-MET pathway has been implicated in B-cell differentiation: in fact, follicular dendritic and stromal cells express HGF while B cells at the level of the germinal center express c-MET.[35,36] In addition, the HGF/c-MET pathway is a potential signaling route in lymphomagenesis since HGF/c-MET are co-expressed in several B-cell malignancies.[37] We previously suggested a potential involvement of the HGF/c-MET pathway in this type of leukemia[38] analyzing this in more detail here. We found high levels of c-MET expression on CLL cells, not paralleled by autocrine HGF secretion. High levels of HGF were, however, produced by BMSC from healthy and CLL subjects and even higher levels by the osteoblast-like cell line MG63: interestingly these cell types strongly enhanced leukemic B-cell survival. It is worth noting that, as previously reported, the levels of HGF, vascular endothelial growth factor and fibroblast growth factor are higher in sera of CLL patients than in normal controls.[20] Accordingly we found moderate concentrations of HGF in sera from two CLL patients analyzed. Altogether these observations suggest that HGF is one of the factors operating in disease progression and dissemination. Although our data suggest that HGF present in patients' sera does not derive from leukemic B cells, we cannot exclude that *in vivo* the intimate BMSC-CLL cross-talk can activate higher HGF autocrine or paracrine release.[39] While some authors stressed that direct contact between stromal cells and CLL was necessary to enhance B-cell survival others observed that viability was also increased in a non-contact transwell culture system.[40,41] We observed a moderate but significant pro-survival effect by using only conditioned media from some mesenchymal cell cultures. The finding that recombinant HGF or conditioned media had less protective effects on CLL cells than co-cultures with MG63 or BMSC suggests that HGF is one of multiple soluble factors mediating increased survival. Nonetheless a critical role of HGF is also confirmed by decreased CLL cell survival once its effects are reverted by

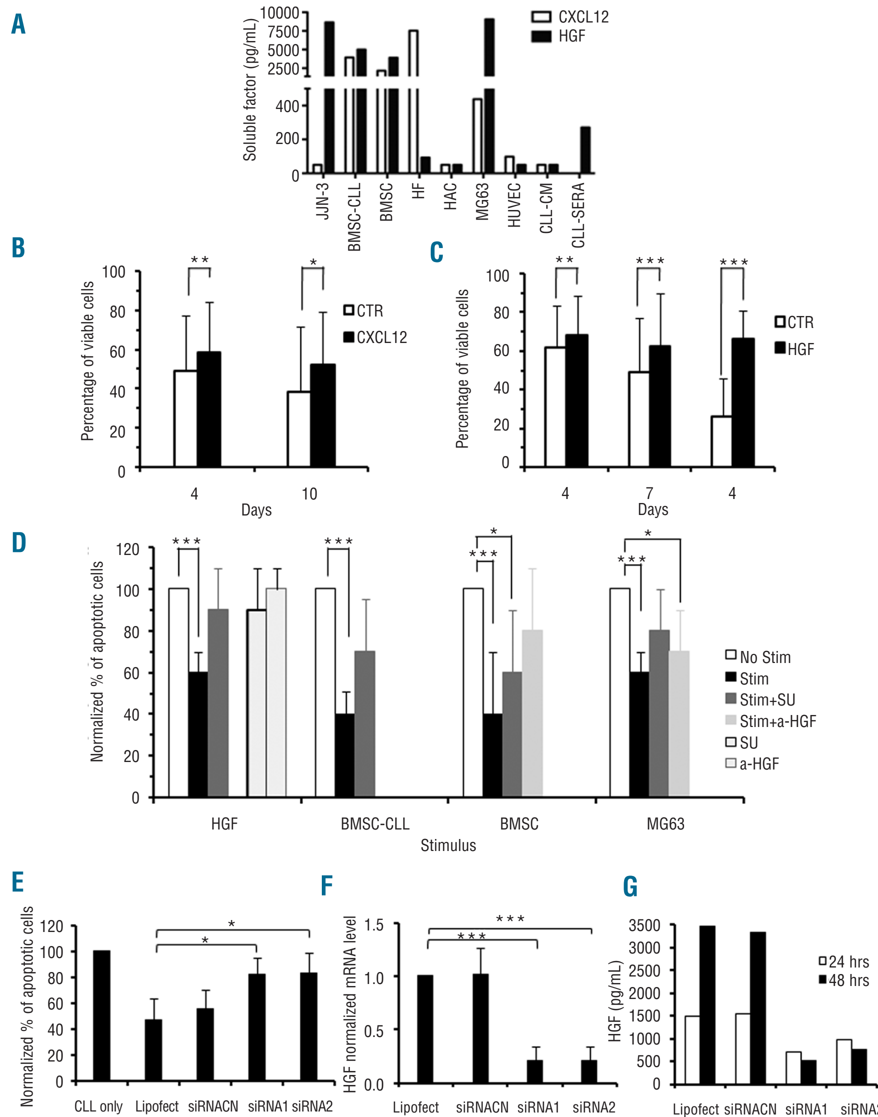

Figure 3. Mesenchymal cells sustaining CLL survival secrete CXCL12 and HGF. (A) CXCL12 is produced by HF, BMSC and MG63, but not by HAC and HUVEC. HGF is produced at high levels by BMSC and MG63 and low levels are present in CLL sera (*n*=2) and in HF-medium. HGF is absent from HAC or HUVEC and from media from CLL cells (CLL-CM, n=4). JJN3: positive control. Values are the mean of two experiments. (B) CXCL12 increased survival of CLL cells, as evaluated by annexin-V/PI. Values are mean±SD n=5 (cases n.1,2,10,11,15) ***P*=<0.01. (C) HGF prolongs CLL cells viability, n=8 (cases n.1,2,4,10,11,15,19,20); ***P*=<0.01. (D) SU11274 (SU) or anti-HGF monoclonal antibody (a-HGF) significantly inhibited the anti-apoptotic effect of HGF (cases n.1,4,10,19,20), or of different stimuli (Stim): co-culture with autologous (BMSC-CLL; cases n.4,10,15,20) or allogeneic BMSC (BMSC; cases n.1,10,19,28) or MG63 (cases n.1,10,11,19,28). Addition of SU or a-HGF alone did not produce results different from unstimulated control values. Data are normalized to the percentage of apoptotic cells in unstimulated cells (No stim). (E) Spontaneous apoptosis of CLL cells (CLL only) was restored after co-culture with HGF-silenced-MG63 (siRNA1, siRNA2) but not with mock-transfected MG63 or when unrelated siRNA (siRNACN) was used. The decrease of HGF transcripts after siRNA transfection was verified by real-time RT-PCR (F) Values in E and F are the mean±SD of three experiments (cases n. 1,10,19) performed in duplicate. (G) Data represent quantification of HGF levels by ELISA in conditioned medium from siRNA-transfected MG63 cultured for 24-48 h in one case (n.1).

the addition of c-MET inhibitor, anti-HGF antibody or by HGF-silencing in co-culture experiments. As already known STAT3 can be directly activated by HGF-c-MET interaction through the SH2 domain of the receptor.[42] In our study a functional link between HGF and STAT3 was supported by the rapid STAT3 phosphorylation observed after HGF treatment (10-20 min), and confirmed by blocking pSTAT3 through the addition of c-MET tyrosine kinase inhibitor. Although slightly variable pSTAT3 basal levels were detected in the analyzed patients, all cases displayed a similar trend in STAT3 phosphorylation upon HGF administration. Moreover MG63- or recombinant HGF-induced survival was antagonized by cpd188 pSTAT3 inhibitor. HGF/c-MET-driven activation of STAT3 should, therefore, be considered along with the already reported PI3K/NFkB/AKT,[43] ERK1/2 and p38[14] signaling transduction pathways. In our studies, however, ERK1/2 was preferentially activated by addition of only CXCL12, while AKT appeared phosphorylated after co-culture with either BMSC or MG63 and after HGF treatment (*Online Supplementary Figure S1*). We tentatively speculate that CXCL12-producing cells, such as fibroblasts, may influence CLL survival through ERK1/2, AKT and possibly JAK/STAT3 signaling while HGF-producing ones could preferentially activate the STAT3 and AKT pathways. Triggering of overlapping pathways could also in part explain the limited additive effect observed when both factors were used in combination (*Online Supplementary Figures S3 and S4*).

At variance with a recent study of the effects induced by co-culture between murine or human cell lines derived from bone marrow and CLL,[41] our intention in this study was to analyze interactions of CLL cells with undifferentiated human BMSC as well as differentiated ones. Indeed, several stromal components within a specific niche may provide different signals to ensure either B-cell lymphopoiesis or long-term survival of fully mature plasma cells. In this light, MG63, representing an osteoblast-like phenotype, may provide new insights into the importance of the osteoblastic niche in the maintenance of the leukemic B-cell clone. In our study, among the various bone marrow components, fibroblasts and osteoblast-like

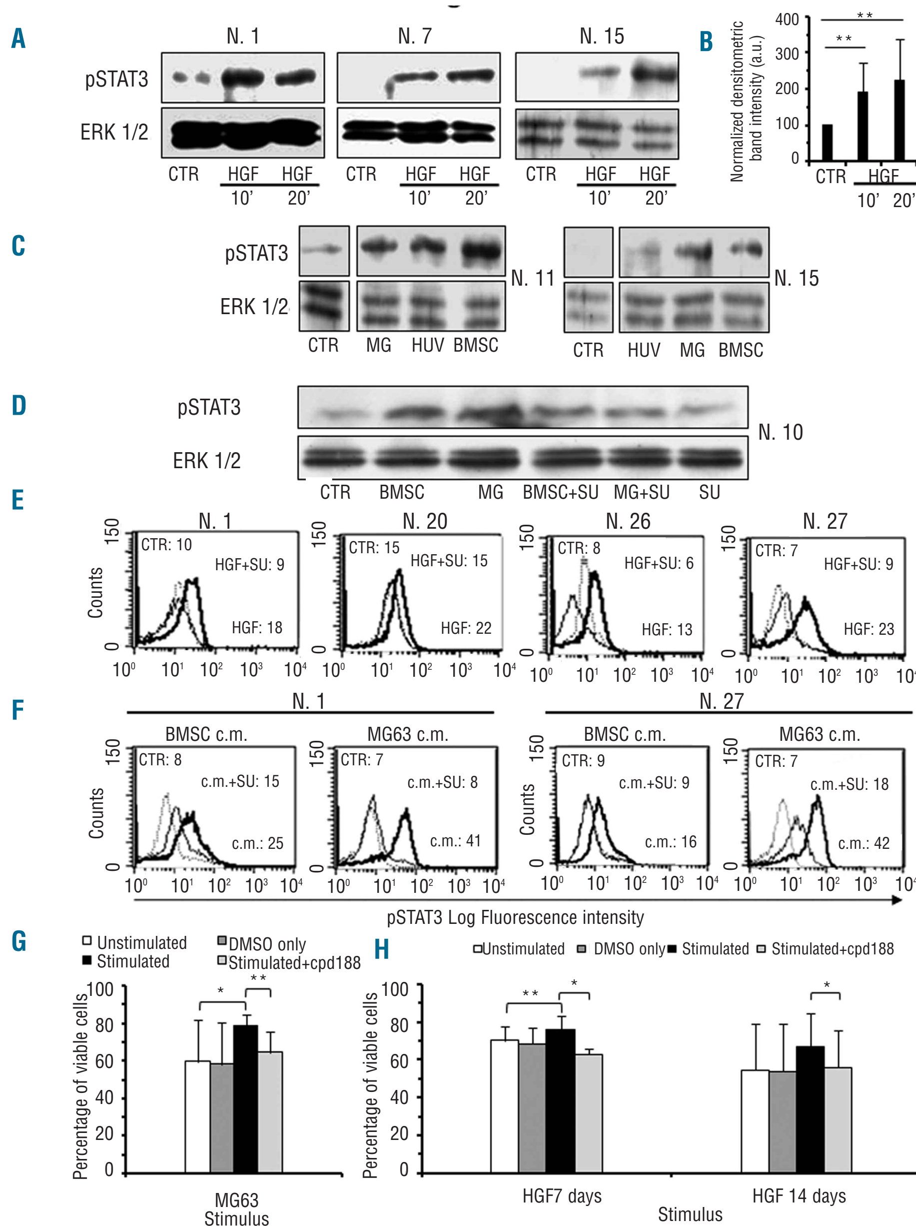

Figure 4. STAT3 phosphorylation in leukemic cells after HGF exposure, or co-cultures with mesenchymal cells or their conditioned media. STAT3 activation and HGF-dependent survival effect were reduced by c-MET and STAT3 inhibitors, respectively. (A) Addition of HGF to CLL cultures activated STAT3 after 10-20 min in three representative cases. Total unphosphorylated ERK1/2 is depicted as the loading controls in all panels. (B) Mean densitometric intensity of pSTAT3 bands in untreated cells (CTR) or 10-20 min after addition of HGF to CLL cell cultures; data are mean±SD of 11 cases tested by western blot analysis (n.1,7,10,11,15,17, 19,20,31,32, 33) after normalization of pSTAT3 band intensities of HGF-treated *versus* untreated cells; $^{**}P<0.01$. (C) STAT3 was also phosphorylated (pSTAT3) after CLL cell co-culture with BMSC and MG63; (D) pSTAT3 was induced after co-culture with MG63 (MG) or BMSC (BMSC), and this effect was inhibited by SU11274 (SU) treatment; CTR: unstimulated CLL cells. (E) Mean fluorescence intensity (mfi) values of pSTAT3, as detected by flow cytometry, in CLL cells cultured in medium alone (CTR: dotted line), with HGF (thick solid line) or with HGF+SU (thin solid line). (F) mfi values of pSTAT3 after 40 min culture with BMSC or MG63 conditioned media (c.m.) with or without SU in two representative cases. (CTR: dotted line; c.m.: thick solid line, c.m.+SU: thin solid line). (G) Spontaneous apoptosis was restored after addition of STAT3 inhibitor (cpd188) to MG63-stimulated CLL co-cultures. Data are mean±SD; n=7 (cases n. 1,2,10,11,27,29, 30). (H) The anti-apoptotic effect of recombinant HGF at 7 or 14 days was significantly reverted by addition of cpd188. Data are mean±SD; n=4 (cases n. 1,2,19,27).

cells appear to be the major supporters of CLL B-cell survival, as depicted in *Online Supplementary Figure S4*. A previous study showed that stromal cells collected by bone biopsy of CLL patients or normal donors were capable of rescuing leukemic B cells from apoptosis and, in agreement with our observations, that this protection was mediated in part by soluble factors.[40] We further confirmed that BMSC from CLL patients supported survival of autologous and allogeneic leukemic cells better than normal BMSC (*Online Supplementary Figure S5*): high levels of HGF and CXCL12 are also produced by BMSC-CLL cells. Indeed aberrant expression of c-MET in leukemic cells and increased HGF sera levels in CLL patients was recently reported,[44] while this paper was under revision.

In conclusion, our results underline that HGF, together with CXCL12, has a pivotal role in the activity of stromal cell feeding of CLL in bone marrow. Moreover, the *in vitro* culture system described here may provide new insights for studying a CLL-supporting bone marrow niche as a promising and feasible target for novel therapeutic strategies.

Authorship and Disclosures

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References

1. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005; 352(8):804-15.
2. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. Blood. 2009;114(16):3367-75.
3. Lagneaux L, Delforge A, Bron D, De Bruyn C, Stryckmans P. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. Blood. 1998;91(7):2387-96.
4. Han T, Barcos M, Emrich L, Ozer H, Gajera R, Gomez GA, et al. Bone marrow infiltration patterns and their prognostic significance in chronic lymphocytic leukemia: correlations with clinical, immunologic, phenotypic, and cytogenetic data. J Clin Oncol. 1984;2(6):562-70.
5. Tsukada N, Burger JA, Zvaifler NJ, Kipps TJ. Distinctive features of "nurselike" cells that differentiate in the context of chronic lymphocytic leukemia. Blood. 2002;99(3):1030-7.
6. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood. 2000;96(8):2655-63.
7. Yin T, Li L. The stem cell niches in bone. J Clin Invest. 2006;116(5):1195-201.
8. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci. 2000;113 (Pt 7):1161-6.
9. Uccelli A, Moretta L, Pistoia V. Immunoregulatory function of mesenchymal stem cells. Eur J Immunol. 2006;36(10): 2566-73.
10. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. Exp Cell Res. 2005;305(1):33-41.
11. Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood. 2005;105(5):2214-9.
12. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood. 2006;107(4):1484-90.
13. Shoham T, Parameswaran R, Shav-Tal Y, Barda-Saad M, Zipori D. The mesenchymal stroma negatively regulates B cell lymphopoiesis through the expression of activin A. Ann N Y Acad Sci. 2003;996:245-60.
14. Tabera S, Perez-Simon JA, Diez-Campelo M, Sanchez-Abarca LI, Blanco B, Lopez A, et al. The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes. Haematologica. 2008; 93(9):1301-9.
15. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006;107(1):367-72.
16. Burger JA, Kipps TJ. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood. 2006;107(5): 1761-7.
17. Deaglio S, Vaisitti T, Aydin S, Bergui L, D'Arena G, Bonello L, et al. CD38 and ZAP-70 are functionally linked and mark CLL cells with high migratory potential. Blood. 2007;110(12):4012-21.
18. Tjin EP, Bende RJ, Derksen PW, van Huijstee AP, Kataoka H, Spaargaren M, et al. Follicular dendritic cells catalyze hepatocyte growth factor (HGF) activation in the germinal center microenvironment by secreting the serine protease HGF activator. J Immunol. 2005;175(5):2807-13.
19. Derksen PW, de Gorter DJ, Meijer HP, Bende RJ, van Dijk M, Lokhorst HM, et al. The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. Leukemia. 2003;17(4):764-74.
20. Aguayo A, Kantarjian H, Manshouri T, Gidel C, Estey E, Thomas D, et al. Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. Blood. 2000;96(6):2240-5.
21. De Totero D, Tazzari PL, Capaia M, Montera MP, Clavio M, Balleari E, et al. CD40 triggering enhances fludarabine-induced apoptosis of chronic lymphocytic leukemia B-cells through autocrine release of tumor necrosis factor-alpha and interferon-gama and tumor necrosis factor receptor-I-II upregulation. Haematologica. 2003;88(2):148-58.
22. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, et al. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med. 2001;344(5):385-6.
23. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell. 2007;131(2):324-36.
24. Giannoni P, Pagano A, Maggi E, Arbico R, Randazzo N, Grandizio M, et al. Autologous chondrocyte implantation (ACI) for aged patients: development of the proper cell expansion conditions for possible therapeutic applications. Osteoarthritis Cartilage. 2005;13(7):589-600.
25. De Totero D, Meazza R, Zupo S, Cutrona G, Matis S, Colombo M, et al. Interleukin-21 receptor (IL-21R) is up-regulated by CD40 triggering and mediates proapoptotic signals in chronic lymphocytic leukemia B cells. Blood. 2006;107(9):3708-15.
26. Bau B, McKenna LA, Soeder S, Fan Z, Pecht A, Aigner T. Hepatocyte growth factor/scatter factor is not a potent regulator of anabolic and catabolic gene expression in adult human articular chondrocytes. Biochem Biophys Res Commun. 2004;316(4):984-90.
27. Grugan KD, Miller CG, Yao Y, Michaylira CZ, Ohashi S, Klein-Szanto AJ, et al. Fibroblast-secreted hepatocyte growth factor plays a functional role in esophageal squamous cell carcinoma invasion. Proc Natl Acad Sci USA. 2010;107(24):11026-31.
28. Burger JA, Burkle A. The CXCR4 chemokine receptor in acute and chronic leukaemia: a marrow homing receptor and potential therapeutic target. Br J Haematol. 2007;137(4): 288-96.
29. Xu X, Kasembeli MM, Jiang X, Tweardy BJ, Tweardy DJ. Chemical probes that competitively and selectively inhibit Stat3 activation. PLoS One. 2009;4(3):e4783.
30. Nishio M, Endo T, Tsukada N, Ohata J, Kitada S, Reed JC, et al. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. Blood. 2005; 106(3):1012-20.
31. Pedersen IM, Kitada S, Leoni LM, Zapata JM, Karras JG, Tsukada N, et al. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. Blood. 2002;100(5):1795-801.
32. Burger JA, Peled A. CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. Leukemia. 2009;23(1):43-52.
33. Boros P, Miller CM. Hepatocyte growth factor: a multifunctional cytokine. Lancet. 1995;345(8945):293-5.
34. Naldini L, Vigna E, Narsimhan RP, Gaudino G, Zarnegar R, Michalopoulos GK, et al. Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET. Oncogene. 1991;6(4):501-4.
35. Skibinski G, Skibinska A, James K. The role of hepatocyte growth factor and its receptor c-met in interactions between lymphocytes and stromal cells in secondary human lymphoid organs. Immunology. 2001;102(4):506-14.
36. van der Voort R, Taher TE, Keehnen RM, Smit L, Groenink M, Pals ST. Paracrine regulation of germinal center B cell adhesion through the c-met-hepatocyte growth factor/scatter factor pathway. J Exp Med. 1997;185(12):2121-31.
37. Tjin EP, Groen RW, Vogelzang I, Derksen PW, Klok MD, Meijer HP, et al. Functional analysis of HGF/MET signaling and aberrant HGF-activator expression in diffuse large B-cell lymphoma. Blood. 2006;107(2): 760-8.
38. De Totero D, Giannoni P, Scaglione S, Quarto R, Narcisi R, Parodi M, et al. Chronic lymphocytic leukemic (CLL) B cells express the HGF receptor (c-MET) and are supported in their survival by HGF-producing mesenchymal stromal cells. Haematologica. 2010;95(Suppl. 2):316.
39. Ding W, Nowakowski GS, Knox TR, Boysen JC, Maas ML, Schwager SM, et al. Bi-directional activation between mesenchymal stem cells and CLL B-cells: implication for CLL disease progression. Br J Haematol. 2009;147(4):471-83.
40. Kay NE, Shanafelt TD, Strege AK, Lee YK, Bone ND, Raza A. Bone biopsy derived marrow stromal elements rescue chronic lymphocytic leukemia B-cells from spontaneous and drug induced cell death and facilitates an "angiogenic switch". Leuk Res. 2007;31(7):899-906.
41. Kurtova AV, Balakrishnan K, Chen R, Ding W, Schnabl S, Quiroga MP, et al. Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance. Blood. 2009;114(20): 4441-50.
42. Gentile A, Trusolino L, Comoglio PM. The Met tyrosine kinase receptor in development and cancer. Cancer Metastasis Rev. 2008;27(1):85-94.
43. Edelmann J, Klein-Hitpass L, Carpinteiro A, Fuhrer A, Sellmann L, Stilgenbauer S, et al. Bone marrow fibroblasts induce expression of PI3K/NF-kappaB pathway genes and a pro-angiogenic phenotype in CLL cells. Leuk Res. 2008;32(10):1565-72.
44. Eksioglu-Demiralp E, Akdeniz T, Bayik M. Aberrant expression of c-met and HGF/c-met pathway provides survival advantage in B-chronic lymphocytic leukemia. Cytometry B Clin Cytom. 2010;80(1):1-7.