# 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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## Online Supplementary Design and Methods

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

Following staining with the primary anti-human-CXCR4 or rabbit anti-human-c-MET monoclonal antibody cells were incubated at 4° C for 30 min. After two washes with phosphate-buffered saline (PBS) + 2% fetal bovine serum (FBS), cells were stained with a secondary goat anti-mouse IgG-FITC monoclonal antibody (Immunotools) or with a goat anti-rabbit IgG-alexafluor-488 monoclonal antibody (Molecular Probes, Oregon, USA) for 30 min more. Cells were washed, resuspended and analyzed by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA).

### Primary cell cultures and cell lines used

Human articular chondrocytes (HAC) were obtained from healthy cartilage specimens. Human skin fibroblasts (HF) were derived from skin biopsies; cells from the MG63 osteoblast-like cell line were from the ICLC cell bank (Genova, Italia; www.iclc.it). Human trabecular bone cells (TBMC) were obtained from extensive washings with PBS of morcellized trabecular bone samples from patients undergoing arthroplasty. Human HAC, HF, TBMC and MG63 cells were all cultured in 10% FBS-supplemented Coon's modified Ham's F12 medium. Human umbilical vein endothelial cells (HUVEC, kindly provided by Dr. N. Ferrari, IST, Genoa, Italy) were cultured on 1% gelatin-coated petri dishes in M199 medium (Sigma, Milan, Italy) with 10 ng/mL aFGF, 10 ng/mL bFGF, 10 ng/mL EGF (Peprotech EC, London, UK), 100 µg/mL heparin (Sigma), and 1 µg/mL hydrocortisone (Sigma).

### Gene chip microarray analysis

Data were downloaded from Array Express (http://www.ebi. ac.uk/microarray-as/ae/) and analyzed in silico with Bioconductor (open source Version 1.2.0; http://www.bioconductor.org/docs/faq/) for background subtraction and normalization (series accession numbers detailed in Online Supplementary Table S2). Genomic data of mesenchymal stem cells were obtained from bone marrow aspirate mRNA samples of three healthy donors, assessed on an Agilent BioAnalyzer and converted into CEL files using Microarray Analysis Suite version 5 (MAS 5, Affymetrix).

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

Qualitative RT-PCR reactions were performed at 95°C for 3 min; 30 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 40 sec, and a final step at 72°C for 7 min. Amplified products were resolved on agarose and photographed under UV light. Samples of cDNA were amplified with the RealMasterMix SYBR ROX 2.5X (5'-Prime) in an Eppendorf Mastecycler Realplex2 apparatus. Results were normalized to the levels of the calibrator gene, GAPDH. Real-time PCR runs were performed in quadruplicate for each sample and the specificity of the reaction products was counterchecked by the analysis of the melting curve.

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

Aliquots of MG63 ($3\times10^4$ cells/well) were seeded in 24-well plates in standard culture medium and allowed to adhere overnight. After washing with PBS, medium was replaced with antibiotic-free RPMI+5% fetal calf serum. The desired short-interfering RNA (siRNA) were diluted in OptiMem® (Invitrogen), containing Lipofectamine 2000 (Invitrogen), and incubated for 30 min at room temperature. Transfections were then performed using 100 µL/well of the final siRNA solution in a final volume of 600 µL.

**Online Supplementary Table S1.** Patients' clinical data.

| Case | Gender | Age (years) | Clin. stage (RAI) | Therapy | CD38 | ZAP70 | IgVH | c-MET |
|------|--------|-------------|-------------------|---------|------|-------|------|-------|
| 1 | Male | 80 | I | no | + | + | u | 85 |
| 2 | Male | 70 | II | no* | + | + | u | 97 |
| 3 | Female | 68 | II | no* | + | + | u | 87 |
| 4 | Male | 69 | II | no^ | - | + | m | 24 |
| 5 | Male | 68 | II | no | - | - | u | 65 |
| 6 | Male | 73 | 0 | no | - | + | m | 83 |
| 7 | Female | 62 | I | no | - | + | m | 81 |
| 8 | Male | 58 | I | no | + | + | u | nd |
| 9 | Female | 68 | IV | no * | - | - | m | 50 |
| 10 | Male | 83 | 0 | no | + | - | m | 83 |
| 11 | Male | 77 | I | no | + | - | m | 94 |
| 12 | Female | 82 | I | no | - | nd | nd | 54 |
| 13 | Male | 70 | I | no | - | + | m | 58 |
| 14 | Male | 76 | 0 | no | - | + | m | nd |
| 15 | Female | 59 | I | no | - | - | m | 84 |
| 16 | Female | 65 | 1 | no | - | - | m | 88 |
| 17 | Female | 77 | 0 | no | - | - | m | 62 |
| 18 | male | 68 | 0 | no | + | + | m | 25 |
| 19 | Male | 78 | 0 | no | - | + | m | 18 |
| 20 | Male | 71 | 0 | no | + | - | m | 74 |
| 21 | Male | 75 | II | no | + | + | m | nd |
| 22 | Male | 67 | 0 | no | + | + | m | 87 |
| 23 | Female | 68 | I | no | + | + | u | 89 |
| 24 | Male | 73 | II | no | + | nd | m | 74 |
| 25 | Female | 65 | 0 | no | - | + | m | 54 |
| 26 | Male | 81 | II | no* | - | + | u | 85 |
| 27 | Female | 81 | 0 | no | + | - | nd | 45 |
| 28 | Male | 74 | 0 | no | + | - | m | 50 |
| 29 | Female | 81 | 0 | no | - | - | m | 90 |
| 30 | Female | 79 | I | no* | + | + | u | 54 |
| 31 | Male | 61 | 0 | no | + | + | u | 76 |
| 32 | Female | 61 | I | no | - | nd | m | 92 |
| 33 | Male | 75 | 0 | no | - | - | m | 90 |

*All patients were out of treatment when enrolled for this study (Therapy: no); some of them subsequently received treatment (*: Chlorambucil; ^: Rituximab) and were reconsidered only when out of treatment for at least 6 months; CD38+: >30%; ZAP70+: >25%; IgVH: u: unmutated, m: mutated >2%; nd: not determined. c-MET expression (%) in CLL cells of the patients was assessed by flow cytometry with a specific antibody (C12, SantaCruz Biotechnology, Inc. CA, USA) on purified CLL cells (>90% CD19+/CD5+). Two-color staining with anti-c-MET (FITC)/-CD19 (PE) and anti-c-MET (FITC)/-CD3(PE) was performed in selected cases to confirm that c-MET was negative on any residual T cells present; nd: not determined.*

**Online Supplementary Table S2.** Accession numbers for the array expression series.

| Cell type | Array accession N. | CEL File N. |
|-----------|--------------------|-------------|
| Chondrocytes | E-GEOD-10575 | GSM266852 |
| | | GSM266853 |
| Foreskin fibroblasts | E-GEOD-4217 | GSM96262 |
| | | GSM96263 |
| | | GSM96264 |
| HUVEC | E-GEOD-9677 | GSM244647 |
| | | GSM244649 |
| | | GSM244651 |
| | | GSM244653 |
| MG63 Osteosarcoma line | E-MTAB-37 | MG-63_SS275749_HCHP-167951 |
| | | MG-63_SS275750_HCHP-167952 |
| | | MG-63_SS275751_HCHP-167953 |

**Online Supplementary Table S3.**

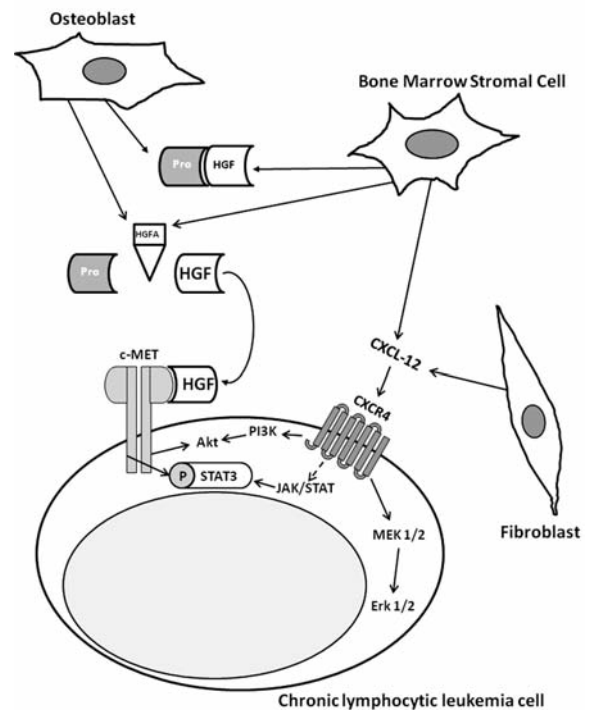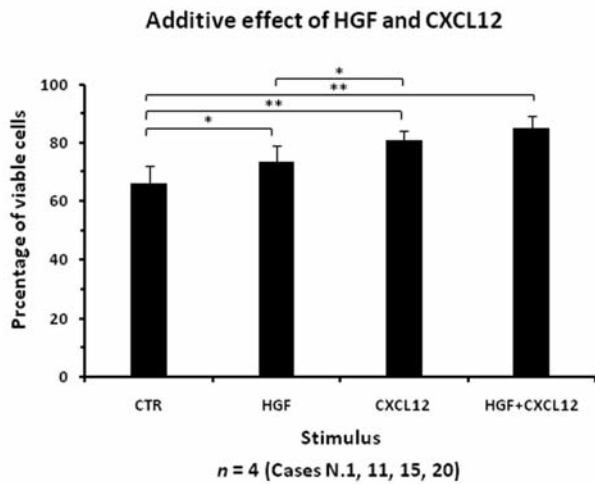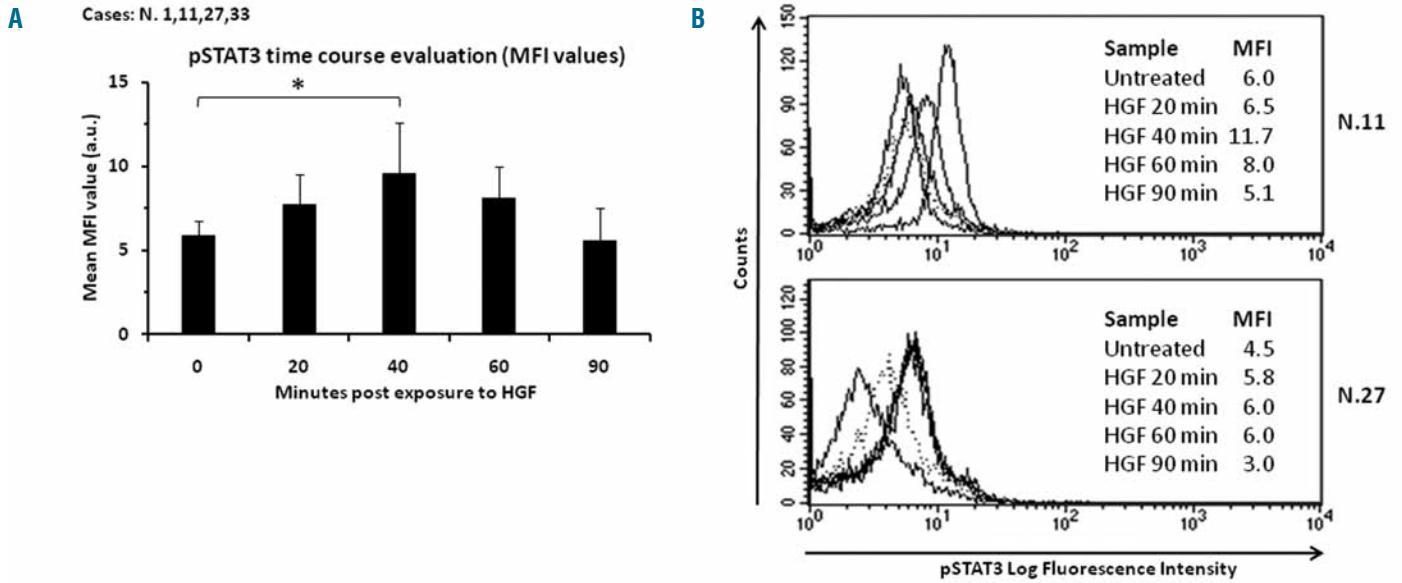| AffyID | Genes | Description |
|--------|-------|-------------|
| 201148 | TIMP3 | TIMP metallopeptidase inhibitor3 (dystrophy, pseudoinflammatory) |
| 203666 | CXCL12 | Chemokine (C-X-C motif)ligand 12 (stromal cell derived factor-1) |
| 204653 | TFAP2A | Transcription factor AP-2 alpha (activating enhancer binding protein 2alpha) |
| 207057 | SLC16A7 | Solute carrier family member 16, member 7(monocarboxylic acid transporter 2) |
| 208900 | TOP1 | Topoisomerase (DNA) 1 |
| 209763 | CHRDL1 | Chordin-like 1 |
| 209960 | HGF | Hepatocyte growth factor (hepatopoietin A, scatter factor) |
| 213689 | RPL5 | Ribosomal protein L5 |
| 214600 | TEAD1 | TEA domain family member 1 (SV40 transcriptional enahncer factor) |
| 215695 | GYG2 | Glycogenin 2 |
| 223940 | MALAT1 | Metastasis associated lung adenocarcinoma transcript 1 |
| 227657 | RFN150 | Ring finger protein 150 |
| 227952 | LOC40211 | Hypothetical LOC402110 |
| 238013 | PLEKHA2 | Pleckstrin homology domain, family A (phosphoinositide binding specific) member 2 |
| 239218 | PDE1C | Phosphodiesterase 1C, calmodulin dependent |
| 239367 | BDNF | Brain-derived neurotrophic factor |

Online Supplementary Table S4. Enhancement of cell viability by HGF.

| CXCL12 Administration: raw data of Figure 3B | | | | |
|---|---|---|---|---|
| | Percentage of viable CLL cells (Annexin V/PI determination) | | | |
| | 4 Days | | 10 Days | |
| Case N. | CTR | +CXCL12 | CTR | +CXCL12 |
| 1 | 22 | 36 | 15 | 30 |
| 2 | 39 | 49 | 7 | 40 |
| 10 | 74 | 87 | 68 | 77 |
| 11 | 25 | 36 | 20 | 28 |
| 15 | 84 | 86 | 80 | 85 |
| Mean | 48,80 | 58,80 | 38,00 | 52,00 |
| SD | 28,53 | 25,84 | 33,46 | 27,01 |

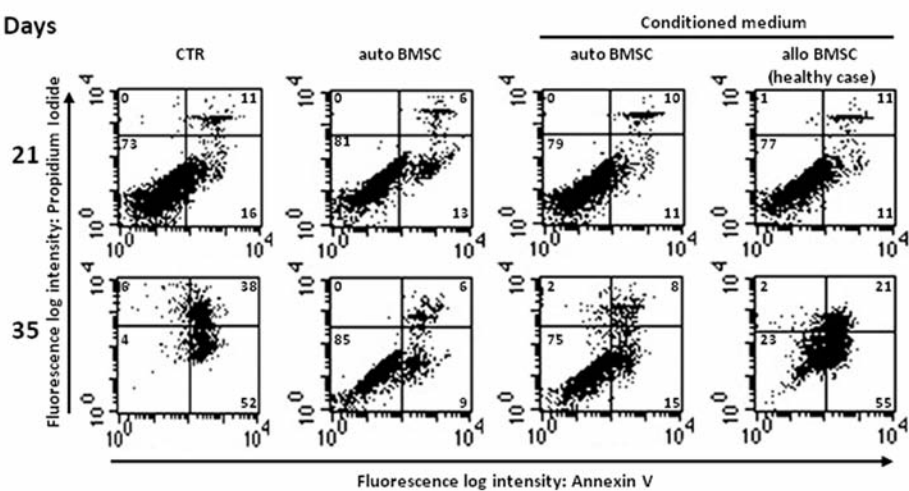| HGF Administration: raw data of Figure 3C | | | | | | |
|---|---|---|---|---|---|---|
| | Percentage of viable CLL cells (Annexin V/PI determination) | | | | | |
| | 4 Days | | 7 Days | | 14 days | |
| Case N. | CTR | +HGF | CTR | +HGF | CTR | +HGF |
| 1 | 41 | 42 | 9 | 16 | 8 | 53 |
| 2 | 40 | 50 | 25 | 38 | 7 | 62 |
| 4 | 75 | 77 | 62 | 68 | 43 | 64 |
| 10 | 76 | 82 | 71 | 85 | 53 | 85 |
| 11 | 31 | 40 | 18 | 42 | 15 | 45 |
| 15 | 89 | 91 | 79 | 88 | 24 | 59 |
| 19 | 64 | 72 | 52 | 68 | 10 | 76 |
| 20 | 78 | 89 | 77 | 93 | 50 | 86 |
| Mean | 61,75 | 67,88 | 49,13 | 62,25 | 26,25 | 66,25 |
| SD | 21,51 | 20,86 | 27,99 | 27,59 | 19,49 | 14,83 |

Online Supplementary Figure S1. Phosphorylation of STAT3, ERK 1/2 and AKT.

A

Cases: N. 1,11,27,33

pSTAT3 time course evaluation (MFI values)

B

| Sample | MFI |
|---|---|
| Untreated | 6.0 |
| HGF 20 min | 6.5 |
| HGF 40 min | 11.7 |
| HGF 60 min | 8.0 |
| HGF 90 min | 5.1 |

N.11

| Sample | MFI |
|---|---|
| Untreated | 4.5 |
| HGF 20 min | 5.8 |
| HGF 40 min | 6.0 |
| HGF 60 min | 6.0 |
| HGF 90 min | 3.0 |

N.27

pSTAT3 Log Fluorescence Intensity

Additive effect of HGF and CXCL12

Stimulus

n = 4 (Cases N.1, 11, 15, 20)

Online Supplementary Figure S5. Survival of leukemic cells co-cultured with autologous or allogeneic BMSC or their conditioned medium.