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# Long term maintenance of myeloid leukemic stem cells cultured with unrelated human mesenchymal stromal cells ☆☆☆

Sawa Ito <sup>a,\*</sup>, A. John Barrett <sup>a</sup>, Amalia Dutra <sup>b</sup>, Evgenia Pak <sup>b</sup>,  
Samantha Miner <sup>a</sup>, Keyvan Keyvanfar <sup>a</sup>, Nancy F. Hensel <sup>a</sup>, Katayoun Rezvani <sup>c</sup>,  
Pawel Muranski <sup>a</sup>, Paul Liu <sup>d</sup>, J. Joseph Melenhorst <sup>a,1,2</sup>, Andre Larochelle <sup>a,1</sup>

<sup>a</sup> Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

<sup>b</sup> Cytogenetics and Microscopy Core, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

<sup>c</sup> University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

<sup>d</sup> Oncogenesis and Development Section, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

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**Abstract** Mesenchymal stromal cells (MSCs) support the growth and differentiation of normal hematopoietic stem cells (HSCs). Here we studied the ability of MSCs to support the growth and survival of leukemic stem cells (LSCs) in vitro. Primary leukemic blasts isolated from the peripheral blood of 8 patients with acute myeloid leukemia (AML) were co-cultured with equal numbers of irradiated MSCs derived from unrelated donor bone marrow, with or without cytokines for up to 6 weeks. Four samples showed CD34<sup>+</sup>CD38<sup>-</sup> predominance, and four were predominantly CD34<sup>+</sup>CD38<sup>+</sup>. CD34<sup>+</sup>CD38<sup>-</sup> predominant leukemia cells maintained the CD34<sup>+</sup>CD38<sup>-</sup> phenotype and were viable for 6 weeks when co-cultured with MSCs compared to co-cultures with cytokines or medium only, which showed rapid differentiation and loss of the LSC phenotype. In contrast, CD34<sup>+</sup>CD38<sup>+</sup> predominant leukemic cells maintained the CD34<sup>+</sup>CD38<sup>+</sup> phenotype when co-cultured with MSCs alone, but no culture conditions supported survival beyond 4 weeks. Cell cycle analysis showed that MSCs maintained a higher proportion of CD34<sup>+</sup> blasts in G0 than leukemic cells cultured with cytokines. AML blasts maintained in culture with MSCs for up to 6 weeks engrafted NSG mice with the same efficiency as their non-cultured counterparts, and the original karyotype persisted after co-culture. Chemosensitivity and transwell assays suggest that MSCs provide pro-survival benefits to leukemic blasts through cell–cell contact. We conclude that MSCs support long-term maintenance of LSCs in vitro. This simple and inexpensive

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\* Corresponding author at: 10 Center Drive, Building 10 CRC 3E-5288, Bethesda, MD 20892 USA. Fax: +1 301 480 2664.

E-mail address: [itos2@mail.nih.gov](mailto:itos2@mail.nih.gov) (S. Ito).

<sup>1</sup> Equally contributed to the manuscript.

<sup>2</sup> Current Address: Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19143, USA.

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## Introduction

Acute myeloid leukemia (AML) is the best studied human tumor for cancer stem cell research and has contributed to our understanding of the cancer stem cell model (Dick, 2008; Lapidot et al., 1994; Bhatia et al., 1998). Since leukemic stem cells (LSCs) are considered to be associated with relapse, chemotherapy resistance and overall poor prognosis (Eppert et al., 2011; Gentles et al., 2010), targeting LSCs has been proposed as a promising strategy for cure of AML (Saito et al., 2010, 2013; Misaghian et al., 2009). Immuno-deficient mouse xenotransplantation models are the gold standard assay for LSCs, and SCID leukemia-initiating cells (SL-ICs) are thought to possess the functional and molecular characteristics of LSCs (Lapidot et al., 1994; Bhatia et al., 1998). However SL-ICs in primary human AML are rare and heterogeneous (Eppert et al., 2011; Gentles et al., 2010; Sarry et al., 2011), therefore patient-derived human primary AML samples are not always sufficient to allow hematopoietic reconstitution in xenotransplantation model. Development of an in vitro LSC culture system would facilitate further advances in cancer stem cell research and assist in the development of novel therapeutic targets of LSCs (Kennedy and Barabe, 2008). Mesenchymal stromal cells (MSCs) are known to support the growth and differentiation of normal hematopoietic stem cells (HSCs) (de Lima et al., 2012; Keating, 2012; Robinson et al., 2006) through various molecular signals such as CXCL12-mediated CXCR4 signaling and Wnt-induced  $\beta$ -catenin signaling. Murine stromal cell lines and growth factors have been used to expand primary human AML blasts in vitro, however the leukemic blasts eventually lost their original CD34<sup>+</sup>CD38<sup>-</sup> phenotype in long-term in vitro culture systems (Klco et al., 2013; van Gosliga et al., 2007). We hypothesized that human MSCs may also provide a survival benefit for LSCs since they share similar molecular signatures with normal HSCs (Eppert et al., 2011; Gentles et al., 2010), and MSC co-culture systems can be utilized for long-term maintenance of LSCs in vitro even without growth factors. Furthermore, understanding relationships between AML and its stromal niche is of importance for defining mechanisms of leukemic persistence and preventing leukemic relapse. Here we co-cultured human primary leukemic blasts with unrelated bone marrow (BM) derived human MSCs and characterized the phenotype and function of leukemic blasts and their ability to engraft in a xenotransplantation mouse model.

## Materials and methods

### Primary leukemic samples

Peripheral blood samples were collected from eight patients with AML (mean age 53, range 23–74; Table 1). Written informed consent was obtained from the patients and healthy volunteers in accordance with the Declaration of

Helsinki for the use of samples for research according to the requirements of the Institutional Review Board of the National Heart, Lung, and Blood Institute and MD Anderson Cancer Center. Cells were thawed in human cell culture medium [RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 10% human AB serum (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Life Technologies, Carlsbad, CA)].

### MSC isolation, culture and expansion

After obtaining informed consents, BM aspirates were collected from healthy volunteers in the Department of Transfusion Medicine, National Institutes of Health. The BM aspirates were plated in 75 cm<sup>2</sup> flask in MSC medium consisting of MEM $\alpha$  (Life Technologies, Carlsbad, CA) supplemented with 20% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), and 1% L-glutamine (Life Technologies, Carlsbad, CA). Non-adherent cells were removed after 24 h, and the adherent cells were cultured for approximately 14 days with twice weekly MSC medium changes. The cells were harvested using 0.05% trypsin-EDTA (Life Technologies, Carlsbad, CA) when 70% confluence was achieved and used for further expansion. The cells were plated at a density of  $4 \times 10^3$ /cm<sup>2</sup> in four-layer cell factory flasks (Thermo Scientific Nunc<sup>™</sup> Cell Factory<sup>™</sup> Systems, Waltham, MA) in MSC medium. Serial passages were obtained once the cells reached 70% confluence and subsequently expanded MSCs were harvested and cryopreserved in liquid nitrogen. Passage 4 MSCs were thawed in human cell culture medium and were irradiated with 50 Gy. The cells were then plated at selected density in flat bottom plates one day before co-culture experiments to allow reticular network formation.

### Isolation of primary leukemic cells and co-culture with MSCs

Cells from primary leukemic samples were stained with antibodies to CD34-APC (clone 581, BD Biosciences, San Jose, CA), and lineage antibodies, including CD2 (clone TS1/8, Biolegend, San Diego, CA), CD3 (clone S4.1PB), CD14 (clone clone Tük4), and CD19 (clone SJ25-C1)-Pacific Blue (Invitrogen, Carlsbad, CA), as well as Propidium Iodide (PI: Molecular Probes, Eugene, OR). Lineage negative (Lin<sup>-</sup>) CD34<sup>+</sup> cells were sorted on FACS Aria II cell sorter (BD, Franklin Lakes, NJ) and  $2.5 \times 10^5$  cells were co-cultured with an equal number of irradiated MSCs in 24-well flat bottom plates with or without cytokines (150 ng/ml FLT3-ligand, 150 ng/ml Stem cell factor (SCF), 50 ng/ml Interleukin-3 (IL-3)). In control wells, Lin<sup>-</sup>CD34<sup>+</sup> cells were cultured without MSC support in the presence or absence of the same cytokines. In all wells, culture media were replaced twice weekly. In transwell assays, sorted Lin<sup>-</sup>CD34<sup>+</sup> cells were placed in the transwell insert (Costar Transwell<sup>®</sup> Permeable Supports: 0.4  $\mu$ m

**Table 1** Characteristics of AML patients.

UPN	Age	Sex	AML subtype	Karyotype	Status at sample collection	Leukemia phenotype
UPN1	71	F	AML/MDS	5q-, 7q-, 8+	Relapse	CD34 <sup>+</sup> CD38 <sup>-</sup> predominant
UPN2	74	M	AML/MDS	Normal	Refractory to chemotherapy	CD34 <sup>+</sup> CD38 <sup>-</sup> predominant
UPN3	43	F	AML/MDS	7q-	Evolved from MDS	CD34 <sup>+</sup> CD38 <sup>-</sup> predominant
UPN4	77	F	AML/MPD-PV	t(2;6),del(3),-4	De novo	CD34 <sup>+</sup> CD38 <sup>-</sup> predominant
UPN5	42	M	AML-FLT3ITD	Normal	Relapse	CD34 <sup>+</sup> CD38 <sup>+</sup> predominant
UPN6	23	M	AML/MDS	Complex	Relapse	CD34 <sup>+</sup> CD38 <sup>-</sup> predominant
UPN7	39	M	M5	7q-	Refractory to chemotherapy	CD34 <sup>+</sup> CD38 <sup>+</sup> predominant
UPN8	57	M	AML/MDS	7q-	De novo	CD34 <sup>+</sup> CD38 <sup>+</sup> predominant

Abbreviations: AML, acute myeloid leukemia; LSC, leukemic stem cell; MDS, myelodysplastic syndrome; MPD, myeloproliferative disease; PV, polycythemia vera.

pore size) with or without MSCs plated in the lower compartment.

### Leukemic phenotype and cell cycle analysis

The phenotype of cultured cells was analyzed weekly using fluorescently-conjugated monoclonal antibodies against CD38-FITC (clone IM0775U), CD34-PECy7 (clone 8G12), CD11b-APCCy7 (clone ICRF44), CD123-PECy5 (clone 9F5), and CD45-V500 (clone HI30), in addition to the lineage panel (CD2, CD3, CD14, CD19–Pacific Blue). Cells were also stained with Annexin V-APC (BD Biosciences, San Jose, CA) and PI and the proportion of viable, non-apoptotic cells was evaluated in Annexin V negative and PI negative populations. Admixed CD45 negative MSCs were easily distinguished from the CD45 positive leukemic cells. Stained cells were acquired on a FACS Canto II (BD Biosciences). For cell cycle analysis, at least 1 million viable cells were first stained with CD34-PECy7, CD45-APC (BD Biosciences) for 15 min at room temperature. After washing, the cells were incubated at 37 °C, 5% CO<sub>2</sub> for 45 min in 2 μM Hoechst 33342 solution (Molecular Probes, Eugene, OR). Pyronin Y 1 μg/ml (Polysciences, Inc. Warrington, PA) was added and incubation continued for another 45 min at 37 °C, 5% CO<sub>2</sub>. After washing, PI was added to the cells before acquiring on an LSR II (BD, Franklin Lakes, NJ). All flow cytometry data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR). The fraction of cells in the G<sub>0</sub>, G<sub>1</sub>, S-G<sub>2</sub>-M phases of the cell cycle was determined by gating on cell populations defined by their DNA and RNA contents.

### Transplantation of leukemic cells into immune deficient mice

Non-obese diabetic/severe combined immunodeficiency IL-2R $\gamma$ null (NSG) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained under specific pathogen-free conditions. 6–8 week old mice (2–5 mice/group) were sublethally irradiated (300 cGy) 18–24 h before transplantation. For limiting dilution analysis, specific CD34<sup>+</sup> cell doses of primary leukemic samples ranging from 5 × 10<sup>3</sup> to 5 × 10<sup>5</sup> were injected into at least 5 NSG mice per dose. To evaluate the engraftment capacity of MSC co-cultured cells, CD45<sup>+</sup> leukemic cells were sorted

4–6 weeks after co-culture, and injected into irradiated mice through tail-vein injection. Equivalent numbers of CD34<sup>+</sup> leukemic cells freshly sorted from primary leukemic samples were used as controls. At week 8 after transplantation, mice were sacrificed and BM cells were harvested from both femurs and tibias. Cells were stained with human-CD45-PE, CD34-PECy7, CD38-FITC, CD123-PECy5, CD90-APC, CD45RA- and human leukemia engraftment was defined as a threshold of 0.1% human CD45<sup>+</sup>CD34<sup>+</sup> cells in total viable BM cells. The animal experiments were approved by the Animal Care and Use Committee of the National Institutes of Health.

### Cytogenetic analysis and spectral karyotyping (SKY)

Samples from the original uncultured Lin<sup>-</sup>CD34<sup>+</sup> sorted leukemic blasts, as well as from Lin<sup>-</sup>CD45<sup>+</sup> leukemic blasts sorted 4–6 weeks after MSC co-culture or 2 months after NSG transplantation were used for metaphase preparations. After mitotic arrest with colcemid (0.015 μg/ml, 2–4 h) (GIBCO, Gaithersburg, MD) and hypotonic treatment (0.075 mol/l KCl, 20 min, 37 °C), the cells were fixed with methanol–acetic acid (3:1) (Schrock et al., 1996). Spectral karyotyping (Applied Spectral Imaging INC, Carlsbad, CA) and DAPI banding techniques were performed to identify structural and numerical chromosome aberrations and clonal evolution during culture. Clones were defined and karyotypes were designated according to the ISCN (2009) (Shaffer et al., 2009).

### Chemosensitivity assay to cytarabine

Lin<sup>-</sup>CD34<sup>+</sup> leukemic blasts were first labeled with CFSE (Vybrant® CFDA SE Cell Tracer Kit, Life Technologies, NY) according to the manufacturer's instructions. CFSE-labeled CD34<sup>+</sup> blasts (2 × 10<sup>4</sup> cells) were co-cultured with or without an equal number of irradiated MSCs (2 × 10<sup>4</sup> cells) in 96 well flat bottom plates. Serial dilutions of cytarabine (Bedford Laboratories, OH) from 10 μM to 0.5 μM were added to each well, and the cells were incubated at 37 °C, 5% CO<sub>2</sub> for 48 h. Leukemic blasts were then harvested from each well and the percentage of Annexin V- and PI-negative viable cells was determined in CFSE-labeled cells using FACS Fortessa. Relative viability was calculated using the formula: % relative viability =

$100 - (a / b \times 100)$  where  $a$  is the percentage of viable cells with or without MSCs in different doses of cytarabine and  $b$  is the percentage viable cells co-culture with MSCs in the absence of cytarabine.

### Statistical analysis

Data were analyzed with Prism Version 5.04 (GraphPad Software, Inc. La Jolla, CA). Results were considered statistically significant if a  $p$  value was found to be less than 0.05 based on one-way ANOVA analysis and paired  $t$ -test. The frequency of LSCs was calculated using L-Cal software (StemSoft Software Inc., Vancouver, CA).

## Results

### Primary leukemic cell surface phenotypes are heterogeneous

We characterized surface markers of eight AML blasts on the basis of CD34 and CD38 expression within the lineage (CD2/CD3/CD14/CD19) negative population. Primary leukemic samples were phenotypically heterogeneous as previously reported (Eppert et al., 2011; Sarry et al., 2011). Based on the proportions of cells co-staining for CD34 and CD38, we classified leukemic samples as CD34<sup>+</sup>CD38<sup>-</sup> predominant leukemias ( $n = 4$ ) and CD34<sup>+</sup>CD38<sup>+</sup> predominant leukemias ( $n = 4$ ) (Supplementary Fig. 1). The latter group included both CMP- and GMP-like leukemias (Supplementary Fig. 2).

### Co-culture with MSCs maintains viable leukemic blasts in vitro

Sorted CD34<sup>+</sup> blasts from primary leukemias were cultured in different conditions as follows: 1) co-culture with MSCs; 2) co-culture with MSCs in a cytokine cocktail consisting of Flt3L, SCF, and IL-3; 3) culture in a cytokine cocktail alone; or 4) culture in human cell culture medium only (medium alone). All leukemic blasts retained significantly higher viable absolute counts after at least 4 weeks when co-cultured with MSCs ( $38 \pm 17 \times 10^3$  cells;  $p = 0.01$ ), MSCs + cytokines ( $193 \pm 108 \times 10^3$  cells;  $p = 0.04$ ), or cytokines ( $163 \pm 83 \times 10^3$  cells;  $p = 0.01$ ) compared to medium alone ( $5 \pm 3 \times 10^3$  cells) except one outlier sample (UPN1) which maintained viable cells even in medium alone (Supplementary Fig. 3). MSCs maintained all CD34<sup>+</sup>CD38<sup>-</sup> predominant leukemic cells viable for up to 6 weeks (Figs. 1A and C). In contrast, CD34<sup>+</sup>CD38<sup>+</sup> predominant leukemic samples remained viable for only 4 weeks when co-cultured with cytokines with or without MSCs (Fig. 1B).

### Co-culture with MSCs maintains the surface phenotype of the primary leukemias in vitro

We evaluated the cell surface phenotype of cultured leukemic cells weekly in each culture condition. CD34<sup>+</sup>CD38<sup>-</sup> predominant leukemic samples ( $n = 4$ ) maintained the original CD34<sup>+</sup>CD38<sup>-</sup> phenotype when co-cultured with MSCs at 6 weeks, in contrast to cultures with MSCs + cytokines

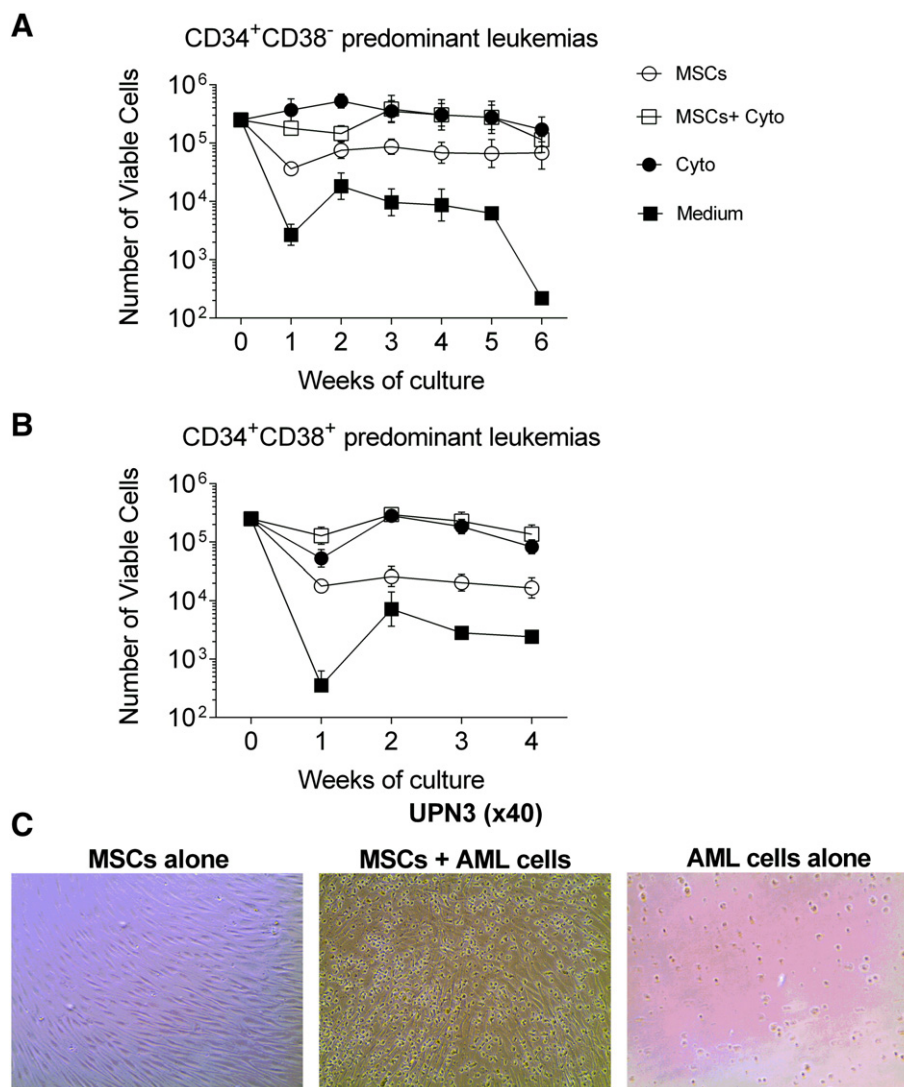
( $p = 0.03$ ), cytokines ( $p = 0.03$ ), or medium alone ( $p = 0.03$ ) which showed rapid decline of the CD34<sup>+</sup>CD38<sup>-</sup> phenotype within two weeks (Fig. 2A). CD34<sup>+</sup>CD38<sup>+</sup> predominant leukemias ( $n = 4$ ) also maintained their CD34<sup>+</sup>CD38<sup>+</sup> phenotype when co-cultured with MSCs at 2 weeks compared with MSCs + cytokines ( $p = 0.05$ ), cytokines ( $p = 0.03$ ), or medium alone ( $p = 0.03$ ) (Fig. 2B). Absolute numbers of CD34<sup>+</sup>CD38<sup>-</sup> cells were highest in MSCs or MSCs + cytokines conditions in all CD34<sup>+</sup>CD38<sup>-</sup> predominant leukemias (Fig. 2C). Similarly, absolute numbers of CD34<sup>+</sup>CD38<sup>+</sup> cells were highest in MSCs or MSCs + cytokines conditions in all CD34<sup>+</sup>CD38<sup>+</sup> predominant leukemias (Fig. 2D). Cells cultured in a cytokine cocktail lost CD34 expression rapidly, suggesting differentiation of the leukemic blasts, while cells co-cultured with MSCs remained undifferentiated as shown in Fig. 2E. The total number of viable leukemic cells and the persistence of the original leukemic phenotype were significantly diminished when leukemic cells were separated from MSCs in a transwell culture system as compared to co-culture conditions allowing direct cell-to-cell contact between MSCs and leukemic blasts (Supplementary Fig. 4).

### Leukemic blasts remained quiescent when co-cultured with MSCs

Cell cycle analysis was performed 1 and 2 weeks after initiation of in vitro culture. A higher proportion of CD45<sup>+</sup>CD34<sup>+</sup> leukemic blasts remained in G0 when co-cultured with MSCs (average  $51.1 \pm 5.1\%$  for all 8 leukemias) compared to cultures in either MSCs + cytokines ( $34.8 \pm 5.1\%$ ;  $p = 0.01$ ) or cytokines ( $34.2 \pm 4.3\%$ ;  $p = 0.05$ , Figs. 3A and B). The G0/G1 ratio was also higher when leukemic cells were co-cultured with MSCs ( $2.0 \pm 0.36$ ) compared to cultures in either MSCs + cytokines ( $0.99 \pm 0.25$ ;  $p = 0.01$ ) or cytokines ( $0.99 \pm 0.28$ ;  $p = 0.05$ ). Absolute numbers of G0 cells were not significantly different with or without MSCs (Figs. 3C and D). These findings indicate that co-culture with MSCs favored quiescence in leukemic blasts and minimized cell cycling compared to culture conditions utilizing cytokines.

### CD34<sup>+</sup>CD38<sup>-</sup> predominant leukemic blasts contain higher frequencies of leukemia initiating cells engrafting in NSG mice

The frequency of leukemia initiating cells in the primary leukemias was evaluated by limiting dilution analysis using two representative samples, UPN1 (CD34<sup>+</sup>CD38<sup>-</sup> predominant leukemia) and UPN6 (CD34<sup>+</sup>CD38<sup>+</sup> predominant leukemia). Eight weeks after injecting a specific dose of sorted leukemic blasts ( $5 \times 10^3$  to  $5 \times 10^5$ ), both leukemias successfully engrafted as defined by more than 0.1% CD45<sup>+</sup>CD34<sup>+</sup> cells in the bone marrow. The calculated frequency of leukemia initiating cells (LIC) was superior in the CD34<sup>+</sup>CD38<sup>-</sup> predominant leukemic sample tested (1 LIC per  $2.7 \times 10^3$ , 95% CI:  $1.6\text{--}4.6 \times 10^3$ ) compared to the CD34<sup>+</sup>CD38<sup>+</sup> predominant leukemic sample studied (1 LIC per  $3.8 \times 10^5$ , 95% CI:  $2.3\text{--}6.4 \times 10^5$ ) ( $p = 0.0003$ ).



**Figure 1** Numbers of viable AML blasts generated during culture. (A) Trend of absolute numbers of viable LSC-like leukemic blasts excluding UPN1 (separately shown in Supplementary Fig. 2). Better viability of leukemic blasts was observed when co-cultured with MSCs, MSCs supplemented with cytokines (MSCs + Cyto) (IL-3, Flt3-ligand, and SCF), cytokines alone (Cyto) in contrast to medium only (Medium). (B) Trend of absolute numbers of viable non-LSC-like leukemic blasts. (C) Microscopic appearance of leukemic blasts co-cultured with MSCs. Representative images for UPN3 (CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemia) indicating preserved morphology of leukemic blasts cultured for 2 weeks in the presence (middle panel) or absence (right panel) of MSCs.

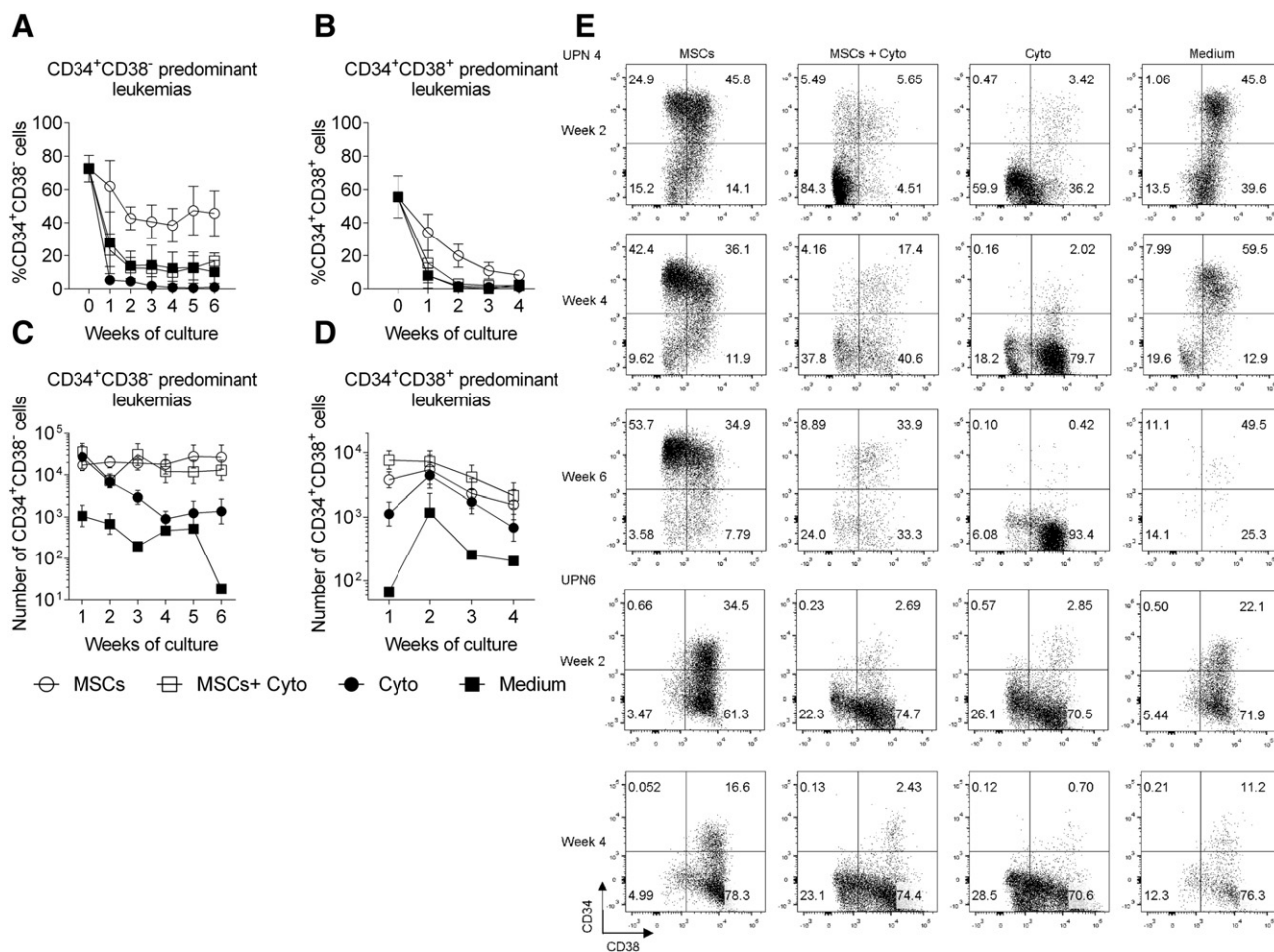
### Leukemic blasts in long-term MSC co-culture systems successfully engraft in NSG mice

We repeated xenotransplantation experiments to confirm the persistence of leukemia initiating cells after MSC co-culture. We cultured UPN1 and UPN6 on MSCs for 6 weeks and 4 weeks respectively, and then injected equivalent numbers of sorted CD45<sup>+</sup> leukemic blasts from MSC co-cultures and non-cultured CD34<sup>+</sup> leukemic blasts. Both leukemias successfully engrafted in NSG mice in 8 weeks achieving equivalent proportions of CD45<sup>+</sup> cell engraftment compared to freshly sorted leukemic cells (UPN1:  $p = 0.54$  and UPN6:  $p = 0.99$  respectively: Fig. 4A). Engrafted cells retained the original phenotypes in both CD34<sup>+</sup> CD38<sup>-</sup> predominant and CD34<sup>+</sup> CD38<sup>+</sup> predominant

samples (Fig. 4B). These results confirmed the maintenance of leukemia initiating cells after long-term in vitro co-culture with MSCs.

### Maintenance of the leukemic clones after co-culture with MSCs

The original chromosomal aberrations found in UPN1 and UPN6 were monitored at different time points: 1) in fresh CD34<sup>+</sup> sorted blasts prior to MSC co-culture; 2) in CD45<sup>+</sup> sorted blasts 6 weeks after MSC co-culture; and 3) in CD45<sup>+</sup> blasts sorted from the bone marrow of NSG mice 8 weeks after transplantation. The chromosomal abnormalities in the original samples persisted after MSC co-culture and



**Figure 2** Phenotype of AML blasts during culture. (A) Percentage of CD34<sup>+</sup>CD38<sup>-</sup> cells in CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemic samples during culture with MSCs, MSCs with cytokines (MSCs + Cyto), cytokines alone (Cyto), or medium alone (Medium). CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemias (average %CD34<sup>+</sup>CD38<sup>-</sup> 72.5%; range 55.6–90.0% at day 0) maintained a higher proportion of CD34<sup>+</sup>CD38<sup>-</sup> cells when co-cultured with MSCs compared to other culture conditions. (B) Percentage of CD34<sup>+</sup>CD38<sup>+</sup> cells in CD34<sup>+</sup> CD38<sup>+</sup> predominant (average %CD34<sup>+</sup>CD38<sup>+</sup> 55.6%; range 30.3–89.9% at day 0) leukemic samples during culture. CD34<sup>+</sup> CD38<sup>+</sup> predominant leukemias maintained a higher proportion of CD34<sup>+</sup>CD38<sup>+</sup> cells when co-cultured with MSCs. (C) Trend of absolute numbers of viable CD34<sup>+</sup>CD38<sup>-</sup> cells in CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemic blasts excluding UPN1 (separately shown in Supplementary Fig. 2). (D) Trend of absolute numbers of viable CD34<sup>+</sup>CD38<sup>+</sup> cells in CD34<sup>+</sup> CD38<sup>+</sup> predominant leukemic blasts. (E) Representative immunophenotype data of CD45<sup>+</sup> leukemic blasts from patient UPN4 (CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemia) and UPN6 (CD34<sup>+</sup> CD38<sup>+</sup> predominant leukemia) during culture. Co-culture with MSCs favored persistence of the original phenotype compared to other culture conditions.

engraftment of MSC co-cultured cells in NSG mice; however other chromosomal aberrations were found (Fig. 5A and B).

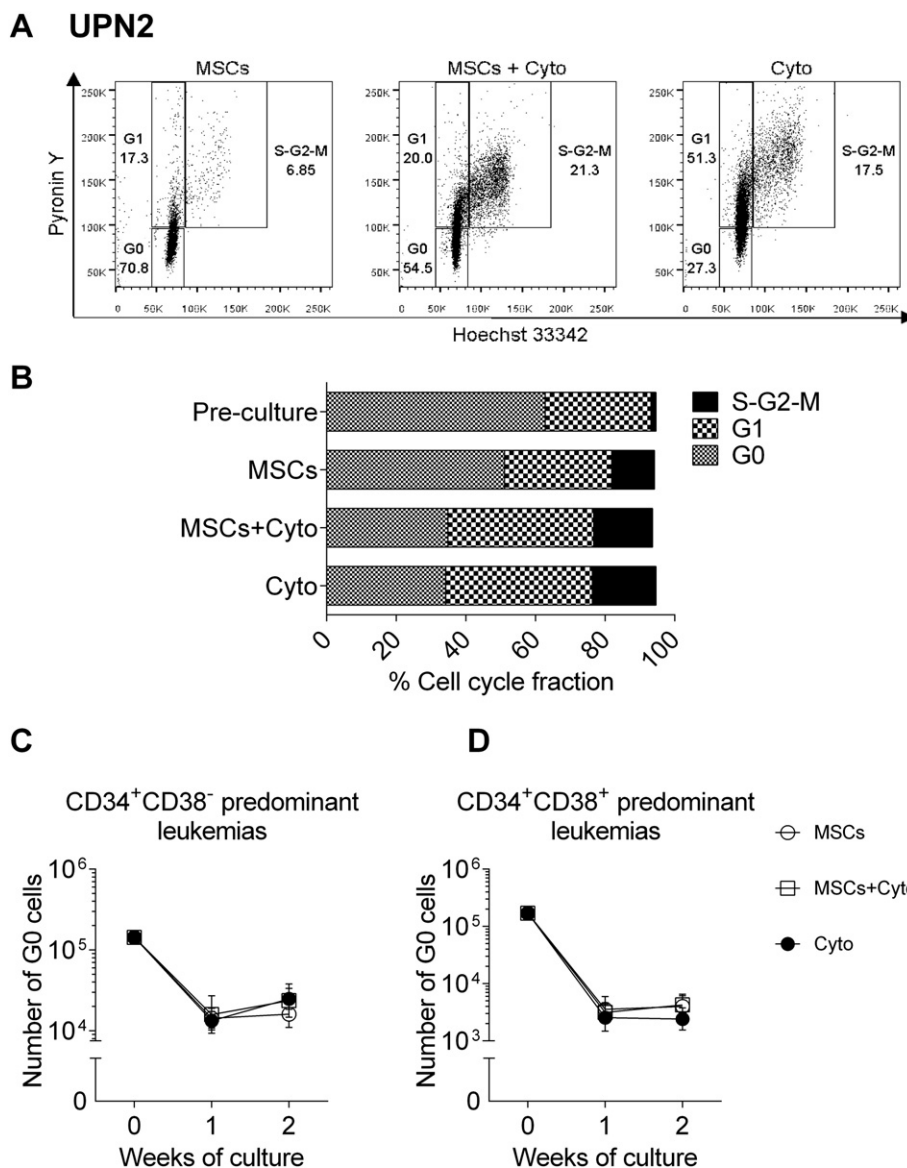
### MSCs promote chemotherapy resistance in leukemic blasts

To evaluate the impact of MSCs on the function of leukemic blasts, we performed a chemosensitivity assay to cytarabine. Six primary leukemic blast samples (Lin<sup>-</sup>CD34<sup>+</sup> cells from CD34<sup>+</sup> CD38<sup>-</sup> predominant (n = 3) and CD34<sup>+</sup> CD38<sup>+</sup> predominant leukemias (n = 3)) were incubated for 48 h with serial dilutions of cytarabine with or without MSCs. The viability of leukemic blasts was significantly higher with less apoptotic cells when co-cultured with MSCs (p = 0.0009; Fig. 6). This finding indicates that MSCs provide

pro-survival benefit, protecting primary leukemic cells from chemotherapy.

### Discussion

BM-derived MSCs represent part of the multicellular stromal support that creates a milieu to maintain HSC function and allow growth and differentiation of hematopoietic progenitors. The ease with which MSCs can be expanded in vitro facilitates the study of the interaction of MSCs with hematopoietic progenitors. MSCs have been successfully employed to increase the hematopoietic stem and progenitor cell numbers in cord blood for transplantation (de Lima et al., 2012). A current interest is whether the marrow milieu is also supportive to AML stem cells and represents a

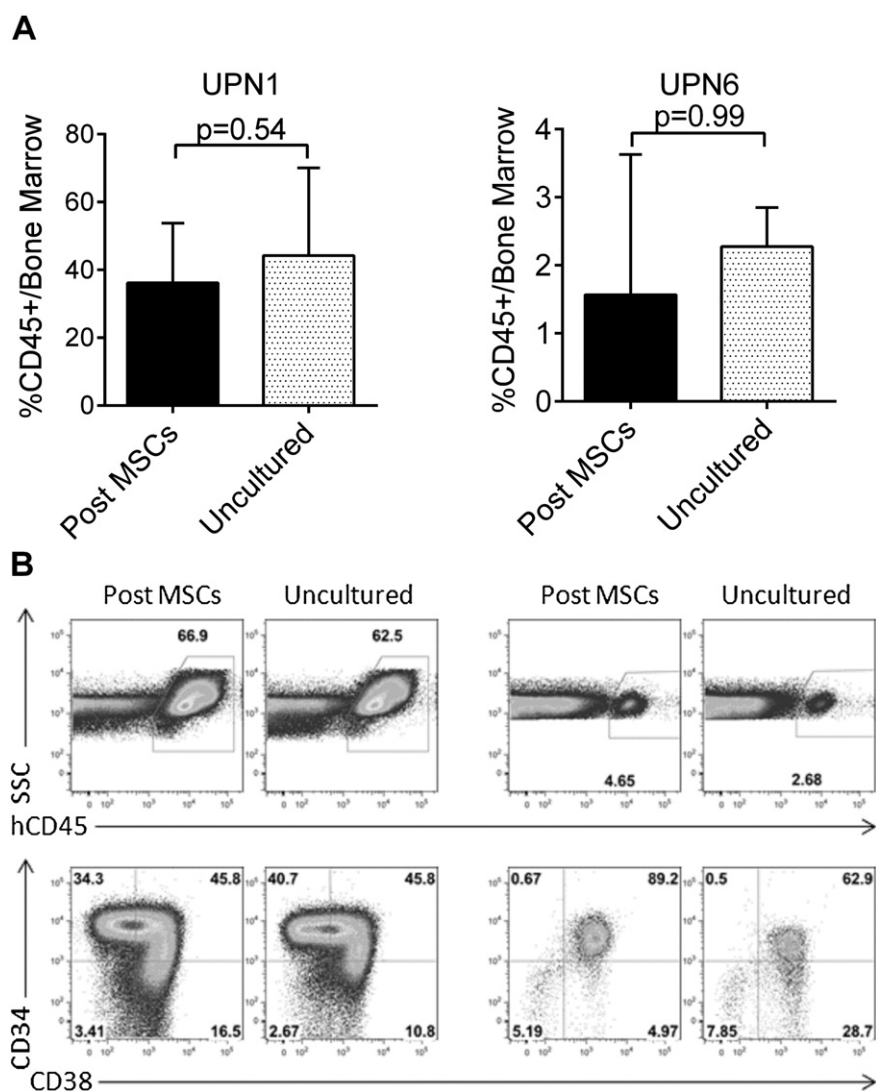


**Figure 3** Cell cycle analysis of AML blasts during culture. (A) Representative cell cycle analysis of UPN2 (CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemia) 1 week after culture with MSCs, MSCs with cytokines (MSCs + Cyto), or cytokines alone (Cyto). (B) Summary of cell cycle analysis before co-culture and 1 week after co-culture (n = 8). A higher proportion of cells remained in G0 when co-cultured with MSCs compared to other culture conditions using cytokines. (C) Trend of absolute numbers of CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemic cells in the G0 phase of the cell cycle. (D) Trend of absolute numbers of CD34<sup>+</sup> CD38<sup>+</sup> predominant leukemic cells in the G0 phase of the cell cycle.

sanctuary for persistence of LSCs after remission. Understanding the nature of the stroma-LSC interactions would inform more effective therapy to eliminate residual leukemia persisting after induction chemotherapy. Furthermore an in vitro stromal/LSC co-culture system that replicates favorable conditions for LSC persistence would serve as a more relevant model for testing agents active against LSCs.

In this study, we demonstrated that unrelated allogeneic human MSCs supported a LSC phenotype in a largely quiescent state in a long-term vitro culture system. We then confirmed the persistence of LIC after 6 weeks co-culture with MSCs using the NSG mouse xenotransplant model. Our results also revealed that MSCs, in contrast to

cocktails of stem cell growth factors or medium alone, support survival of quiescent LSCs with minimal outgrowth of more differentiated leukemic populations. Of interest, for one of 8 leukemic samples studied, LSCs were maintained in culture for up to 6 weeks even without MSC support. Variability between leukemic samples has been described before (Klco et al., 2013). While the mechanisms underlying the atypical in vitro growth pattern of leukemic blasts in this patient remain unclear, we have observed increased telomere length in this sample compared to leukemic cells derived from age-matched patients (unpublished data). The maintenance of viable leukemic blasts in a stromal co-culture system is consistent with the previous findings



**Figure 4** Transplantation of AML blasts in NSG mice. (A) Summary (post MSCs,  $n = 5$  mice; uncultured,  $n = 2$  mice) and (B) representative experiment of human cell engraftment in the bone marrow of NSG mice 8 weeks after transplantation. Both leukemic blasts harvested 4 and 6 weeks after MSC co-culture, respectively, maintained their ability to engraft compared to uncultured blasts derived from the original leukemic samples. The engrafted cells retained the original phenotypes regarding to CD34 and CD38 expression.






observed by van Gosluga et al (van Gosluga et al., 2007) using murine MS5 stromal cell lines and by Klco et al (Klco et al., 2013) using human HS27 stromal cell lines to expand primary AML blasts. However, both groups combined MSCs with growth factors (SCF, IL-3, Flt-3 ligand +/- TPO), and one study demonstrated loss of the CD34<sup>+</sup> cell population after long-term co-culture (van Gosluga et al., 2007). While the distinct stromal cells utilized in each study may account for the differences observed, our data suggest that addition of growth factors during co-culture results in depletion of LSCs by promoting their differentiation in vitro.

We also demonstrated that the protective effect on LSCs requires direct cell-to-cell contact since a transwell culture system failed to replicate the findings in vitro. The nature of the interactions between MSCs and leukemic blasts that favor survival, quiescence and maintenance of the original phenotype remains to be elucidated. Despite the prolonged culture (and in keeping with the

preservation of quiescence) we found that the clonality of the original leukemic population was largely preserved by MSCs. This ability of MSCs to preserve the original leukemic phenotype was observed more significantly in CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemias than CD34<sup>+</sup> CD38<sup>+</sup> predominant leukemias. The non-LSC-like leukemias had two logs fewer leukemia initiating cells than the LSC samples, thus the preferential pro-survival effects of MSCs in vitro may be largely restricted to preserving leukemic stem cells. Further studies to identify the mechanisms of LSC support by MSCs should shed light on the role of MSCs in the marrow milieu.

While remission induction has continued to improve in patients with AML, disease relapse remains a major limitation for cure. We showed that leukemic blasts maintained on MSCs were more resistant to cytotoxicity from cytarabine, a widely used nucleoside analogue to induce remission in AML. Currently allogeneic stem cell

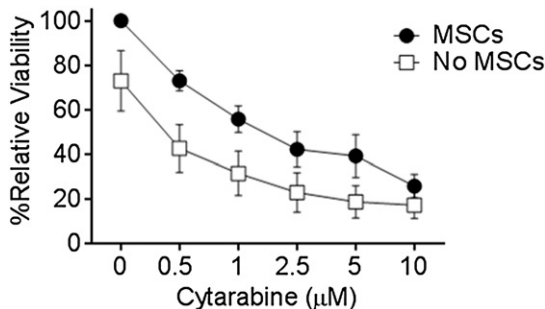


A		UPN1	UPN6
Original CD34+		44,XX,-2,del(5),-7,+8,del(12),der(16)t(16;17),-17,der(18)t(18;7)+del(19),-21 	46,XY,t(2;3) 
Post MSC co-culture		45,XX,del(5),-7,+8,del(12),der(16)t(16;17),-17,der(18)t(18;7) 	Not enough metaphases
Post xeno-transplant CD45+		45,XX,del(5),-7,+8,del(12),der(16)t(16;17),-17,der(18)t(18;7) 	46,XY,t(2;3),del(9) 

B		UPN1	UPN6
Original CD34+		Total cells analyzed 17 Clone 1: 44,XX,-2,del(5),-7,+8,del(12),der(16)t(16;17),-17,der(18)t(18;7)+del(19),-21[7] Clone 2: 47,XX,del(5),-7,+8,del(12),der(18)t(18;7)+del(19)[5] Clone 3: 46,XX,der(2)t(2;16),del(5),-7,+8,der(16)t(16;17),der(18)t(18;7)[3] Clone 4: 41,XX,del(5),-6,-7,+8,-9,del(12),-17,der(18)t(18;7),-21,-22[2]	Total cells analyzed 20 Clone 1: 46,XY,t(2;3)[8] Clone 2: 46,XY,t(2;3),t(11;16)[5] Clone 3: 46,XY,t(1;12),t(2;3),t(2;6),t(13;14)[3] Clone 4: 46,XY,t(2;3),t(2;13),del(14)[2] Clone 5: 47,XY,t(1;12),t(2;3),t(2;6),del(6),der(9)t(9;10),+del(13),del(14)[2]
Post MSC co-culture		Total cells analyzed 25 Clone 1: 46,XX,del(5),-7,+8,del(12),der(16)t(16;17),der(18)t(18;7)[13] Clone 2: 45,XX,del(5),-7,+8,del(12),der(16)t(16;17),-17,der(18)t(18;7)[6] Clone 3: 47,XX,del(5),-7,+8,del(12),der(16)t(16;17),der(18)t(18;7),+del(19)[4] Clone 4: 45,XX,del(5),-7,+8,der(16)t(16;17),-17,der(18)t(18;7)[3]	Not enough metaphases
Post xeno-transplant CD45+		Total cells analyzed 24 Clone 1: 45,XX,del(5),-7,+8,del(12),der(16)t(16;17),-17,der(18)t(18;7)[13] Clone 2: 44,XX,t(1;7),t(2;16),del(5),-7,+8,del(12),-16,der(16)t(16;17),der(18)t(18;7)[3] Clone 3: 45,XX,t(1;7),t(2;16),del(5),-7,+8,del(12),-16,der(16)t(16;17),der(18)t(18;7)[2] Clone 4: 45,XX,t(1;7),t(2;16),del(5),-7,+8,del(12),-16,der(16)t(16;17),der(18)t(18;7)[2] Clone 5: 48,XX,del(5),t(5;10),-7,del(7),+8,del(12),del(14),der(16)t(16;7),der(18)t(18;7)[2] Clone 6: 45,XX,del(5),-7,del(7),+t(8;16),del(12),-16,der(18)t(18;7)[2]	Total cells analyzed 22 Clone 1: 46,XY,t(2;3)[16] Clone 2: 46,XY,t(2;3),del(9)[6]

**Figure 5** Cytogenetic analysis of leukemic blasts. (A) Representative images of spectral karyotype analysis (SKY) and (B) cytogenetic abnormalities from clones found in leukemic blasts. For both UPN1 blasts (CD34<sup>+</sup> CD38<sup>-</sup> predominant leukemia) and UPN6 blasts (CD34<sup>+</sup> CD38<sup>+</sup> predominant leukemia), the original chromosomal abnormalities (upper panels) persisted in CD45<sup>+</sup> blasts sorted 6 weeks after MSC co-culture (middle panels) as well as in CD45<sup>+</sup> blasts sorted from the bone marrow of NSG mice 8 weeks after transplantation (lower panels). Additional chromosomal aberrations were also detected.



**Figure 6** Chemosensitivity of leukemic blasts to cytarabine. Cells from six primary leukemic samples were cultured for 48 h with or without MSCs in the presence of different doses of cytarabine. Data are represented as a relative percentage of viability compared MSC co-cultured blasts. The viability of leukemic blasts was significantly higher when co-cultured with MSCs.

transplantation represents one of the most reliable strategies for eliminating residual disease through a graft-versus-leukemia (GVL) effect. Leukemic cells capable of initiating leukemia in a xenograft model are currently the best descriptor of the cell type responsible for leukemic relapse after chemotherapy. Our in vitro model could be used to study interactions of alloreactive lymphocytes with GVL-like effects on LSCs as well as to explore new agents with specificity for quiescent LSCs. While we show differences in the supportive capacity of MSCs for CD34<sup>+</sup> CD38<sup>-</sup> predominant and CD34<sup>+</sup> CD38<sup>+</sup> predominant leukemias, additional studies will be needed to clarify the nature of MSC interactions with specific AML subtypes, and to correlate LSC maintenance in vitro with relapse probability in vivo.

### Conclusion

Co-culture of leukemic blasts with MSCs represents a simple approach to maintain leukemia initiating cells in

vitro. This culture system will permit exploration of the protective effects of the marrow microenvironment on leukemic stem cells and serve as an improved method to explore therapeutic approaches targeting the leukemic stem cell.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2014.11.007>.

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