Acute myeloid leukemia shapes the bone marrow stromal niche *in vivo*

Alice Pievani,[1] Samantha Donsante,[2*] Chiara Tomasoni,[1] Alessandro Corsi,[2] Francesco Dazzi,[3] Andrea Biondi,[1,4] Mara Riminucci[2] and Marta Serafini[1]*

**AP and SD contributed equally as co-first authors*

[1]Centro Ricerca M. Tettamanti, Department of Pediatrics, University of Milano-Bicocca, Monza, Italy; [2]Department of Molecular Medicine, Sapienza University, Rome, Italy; [3]Department of Hemato-Oncology, Rayne Institute, King's College London, London, UK and [4]Department of Pediatrics, Fondazione MBBM/San Gerardo Hospital, Monza, Italy

Correspondence: MARTA SERAFINI - serafinim72@gmail.com

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Supplementary Methods

Patients and healthy donor samples

Bone marrow (BM) samples of 8 pediatric AML patients at diagnosis and 8 age-matched healthy donors were collected. Mononuclear cells (MNC) were isolated using a Ficoll-Paque^{TM} Plus (GE Healthcare) density gradient separation and used either fresh or after cryopreservation for experiments. The study was approved by the Ethics Committee of San Gerardo Hospital-Monza (BM-NICHE protocol). Informed consent was obtained in accordance with requirements and procedures were carried out in accordance with relevant guidelines and regulations. Clinical and biological patients' features are reported in Supplementary Table 1.

Isolation and culture of BMSC

BM-MNCs were seeded at a density of 2×10^6 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM)–low glucose (Gibco) supplemented with 10% fetal bovine serum (FBS) (Biosera), 1% penicillin-streptomycin (Euroclone) and 1% L-glutamine (Euroclone). Non-adherent cells were removed 24 h after initial plating. The cultures were maintained in basal medium until they reached 70% confluence and then harvested with the use of 0.05% trypsin (Euroclone).

Colony-forming unit-fibroblast assay

The number of clonogenic progenitors was determined by the colony-forming unit-fibroblast (CFU-F) assay. Briefly, BMSC from passage 0 were seeded at clonal density (1.6 cells/cm^2) and maintained for 14 days in basal medium. To enumerate CFU-F, the cells were fixed with methanol, stained with Giemsa solution (Merck KGaA) and scored. The experiment was performed in triplicate for each sample. The clonogenic efficiency was calculated as the number of colonies per 100 initially seeded cells.

Population doubling assay

The population doublings (PD) were calculated for each BMSC sample using the following equation: PDn= PDn–1+[log(C1/C0)]/log2, wherein C0: cells number initially seeded and C1: cells number harvested. Results were expressed as cumulative PD from P1 to P11.

Immunophenotype

BMSC at passage 3 were harvested and stained with phycoerythrin-conjugated or fluorescein isothiocyanate-conjugated monoclonal antibodies specific for CD14 (clone 61D3; eBioscience), CD34 (clone 581; BD Biosciences), CD45 (clone HI30; BD Biosciences), CD90 (clone 5E10;

eBioscience), CD73 (clone AD2; BD Biosciences), CD105 (clone SN6; eBioscience), CD146 (clone P1H12; BD Biosciences), HLA-ABC (clone G46-2.6; BD Biosciences), and HLA-DR (clone G46-6; BD Biosciences). Unstained BMSC were used as negative controls to assess background fluorescence. Flow cytometric analyses were performed with a FACS CantoII (BD Biosciences) instrument, and data were analysed with FACSDiva software (BD Biosciences).

Trilineage differentiation

Adipogenic differentiation was evaluated at P3 by incubating BMSC (200.000 cells/cm^2) with adipogenic induction medium consisting of DMEM-high glucose (Gibco) supplemented with 10% FBS (Biosera), 1 µM dexamethasone (Sigma-Aldrich), 1 µM indomethacin (Sigma-Aldrich), 500 µM 3-isobutyl-1-methylxantine (Sigma-Aldrich), and 10 µg/mL human recombinant insulin (Sigma-Aldrich). Cell cultures were maintained for 21 days in differentiation medium before evaluating differentiation by staining of fat droplets with Oil Red O (Sigma-Aldrich).

Osteogenic differentiation capability of BMSC was assessed at P3 by incubating cells (60.000 cells/cm^2) with osteogenic induction medium consisting of DMEM-low glucose (Gibco), supplemented with 10% FBS (Biosera), 100 nmol/L dexamethasone (Sigma-Aldrich), 10 mM ß-glycerol-phosphate (Sigma-Aldrich), and 0.05 mM 2-phosphate-ascorbic acid (Sigma-Aldrich). Cell cultures were maintained for 21 days in differentiation medium before evaluating the calcium deposition by staining Alizarin Red S (Sigma-Aldrich).

Chondrogenic differentiation was obtained culturing BMSC for 3 weeks as micro-masses in 15 ml polypropylene conical tubes at a density of 300.000 cells/tube in chondrogenic differentiation medium (CDM) consisting of DMEM-high glucose (Gibco), supplemented with ITS^{TM} Premix (BD Biosciences), 1 mM sodium pyruvate (Gibco), 50 µg/mL 2-phosphate-ascorbic acid (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich), 0.1 mM non-essential amino acid solution (Gibco), and 10 ng/mL transforming growth factor (TGF)-β1 (R&D Systems).

To evaluate the capacity of BMSC to generate mineralized cartilaginous matrix, pellets were cultured for 3 weeks in CDM followed by 2 weeks in presence of 7.0 mM β-glycerophosphate and 50 nM thyroxine (without TGFβ1) *(Muraglia A, J Cell Sci 2003).*

RNA isolation and quantitative real-time–polymerase chain reaction

Total RNA was extracted using TRIZOL reagent (Invitrogen), according to manufacturer's protocol. 1 µg of RNA was reversely transcribed using the SuperScript II Reverse Transcriptase (Invitrogen). The cDNA was amplified for specific targets using TaqMan assays on ABI 7900 Real-Time PCR system (Applied Biosystems). The TaqMan probes used are listed in Supplementary Table 2. As reference, housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used.

In vivo transplantation

In vivo experiments were performed in 8- to 10-week-old immunodeficient SCID/beige (CB17/Icr.Cg-Prkdc^{SCID}Lyst^{bg}/Crl) or NSG (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mice from Charles River Laboratories. All animal procedures were performed under license approved by the Italian Ministry of Health (permit number 22/2014-PR).

Transplantation of BMSC on hydroxyapatite/tricalcium phosphate (HA/TCP) carrier was performed as previously reported (*Sacchetti B, Cell 2007*). Briefly, for each transplant 2x10^6 cells were loaded on 40 mg of HA/TCP. Cell/carrier constructs were then transplanted subcutaneously into SCID/beige mice, previous anesthetised with an intramuscular injection of Zoletil 20 (Virbac; 5 mL/g of body weight).

Transplantation of BMSC as unmineralized pellets was performed as previously described (*Serafini M, Stem Cell Rep 2014*). Briefly, after 21 days of *in vitro* differentiation, cartilaginous pellets were transplanted in subcutaneous pouches of anesthetised NSG mice (four samples per mouse) as close as possible to blood vessels.

Animals were sacrificed after 8 weeks and implants harvested for histological analyses.

Histology and Histomorphometry

Cartilaginous pellets and transplants harvested at 8 weeks were fixed with 4% formaldehyde in PBS pH 7.4 and routinely processed for paraffin embedding. Transplants containing bone and bone/ceramic were previous decalcified in 10% EDTA. Four μm thick paraffin sections were stained with Hematoxylin and Eosin (H/E), Sirius Red, and Toluidine Blue and analyzed by either transmitted or polarized light microscopy.

For analysis of bone matrix mineralization, undecalcified ossicles were embedded in poly-methyl-methacrylate (PMMA). Three-micron-thick sections were stained with von Kossa and counterstained with Methylene Blue to distinguish between mineralized and unmineralized bone.

Tartrate-Resistant-Acid-Phosphatase (TRAP) histochemistry was performed by using Sigma Aldrich reagents (Sigma Aldrich) to visualize mono- and multi-nucleated osteoclasts according to the manufacturers' instructions.

Transmitted light and polarized light microscopy images were obtained using Zeiss Axiophot microscope (Carl Zeiss). Digital images were used to perform measurements of bone area and osteoclasts in HA/TCP transplants and adipocytic area in cartilaginous pellets-derived ossicles. H/E and TRAP sections were scanned via Aperio Scan Scope CS (Leica Biosystem Imaging, Nußloch, Baden-Wurttemberg, Germany) and analyzed using the Aperio ImageScope™ program (v12.3.2.8013) to measure Bone Area/Tissue Area (B.Ar/T.Ar), Osteoclast Surface/Mineralized

Surface (OcS/MS) and Adipocytic Area/Marrow Area (Ad.Ar/Ma.Ar) according to the guidelines of the American Society of Bone and Mineral Research (*Dempster DW, J Bone Min Res 2013*) and of the Nomenclature Working group of the Bone Marrow Adipose Society (*Bravenboer N, Front Endocrinol 2020*).

Immunolocalization

Immunolocalization of myeloperoxidase and TER-119 was performed using a rabbit polyclonal anti-mouse MPO antibody (1:300; A0398, Dako) and a rat monoclonal anti-mouse TER-119 antibody (1:50; 550565, BD Pharmingen) with incubation time respectively of 1 and 2 hours at room temperature. Adipocytes were immunolabelled with a rabbit polyclonal anti-human perilipin antibody (1:500, ab3526 Abcam). Sections were incubated for 2 hours at room temperature. Heat mediated antigen retrieval was performed using EDTA buffer pH 7.4.

Immunolocalization of osterix was performed using a rabbit polyclonal anti-human SP7 antibody (1:100; ab22552, Abcam) with an overnight incubation at +4°C. Immunolocalization of osteocalcin and dentin matrix acidic phosphoprotein 1 was performed using a rabbit polyclonal anti-human osteocalcin antibody (1:100; ab93876, Abcam) and a rabbit polyclonal anti-human dmp1 antibody (1:500, LSB11226, LSBio) with incubation time respectively of 30 minutes and 2 hours at room temperature. Heat mediated antigen retrieval was performed using sodium citrate buffer pH 6.

Biotin-conjugated swine anti-rabbit IgG (E0353, Dako) and rabbit anti-rat IgG (E0468, Dako) were used as secondary antibodies. The color reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector).

Total hematopoietic tissue and myeloid/erythroid ratio were determined using the publicly available ImageJ plugin, ImmunoRatio. ImmunoRatio measures the percentage of DAB-stained area over total hematoxylin-stained nuclei area.

Immunofluorescence experiments were performed using anti-human LaminA/C antibody (1:500; ab108595, Abcam) and anti-human CD146 antibody (1:150; ab75769, Abcam). Sections were incubated with primary antibodies overnight at +4°C. Heat mediated antigen retrieval was performed using Tris/EDTA buffer pH 9. Alexa Fluor 488-conjugated goat anti-rabbit (A-11008, Invitrogen) was used as secondary antibody. Nuclei were stained with TOPRO-3 iodide (Invitrogen). Confocal fluorescence images were obtained using the Leica TCS SP5 confocal laser scanning microscopy system (Leica Microsystems).

Statistical analysis

Data were analysed using GraphPad Prism (GraphPad Software). Differences between groups were compared with the Student *t* test. p values ≤ 0.05 were considered statistically significant.

Supplementary References

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Supplementary Table 1

Clinical and biological patients' details.

Patient code	Age (y)	Sex	AML type	% Blasts in BM	Molecular status	Karyotype
AML1	17	M	M1, de novo	60	none*	46,XY[15]
AML2	9	F	M4, de novo	90	MLL-ELL	46,XX,t(11;19)(q23;p13)[18]/46,XX[2]
AML3	3	M	M2, de novo	80	NUP98-NDS1 - t(5;11)	46,XY[20]
AML4	13	F	M2, de novo	80	AML1-ETO, cKIT mut	46,XX,t(3;7)(?q25;?q22), t(8;21)(q22;q22)[2]/46,idem,del(9)(q12 q22)[18]
AML5	13	M	M4, de novo	60	MLL-AF9 – t(9;11)	46,XY,t(9;11)(p21;q23)[25]
AML6	1	M	M5a, de novo	80	MLL-AF10	46,XY,t(10;11)(p12;q23),der(14)t(1;14)(q?21;q11)[20]
AML7	8	F	M4, de novo	85	FLT3-ITD, DEK-CAN - t(6;9)	47,XX,+8[18]/47,idem,iso(13)(q11)[2]
AML8	14	M	M1, de novo	95	none	47,XY,+?13[11]/46,XY[9]

*None, negative for mutations and translocations analysed

Supplementary Table 2

RT-PCR primers.

Protein	*Gene symbol*	*Primer for RT-PCR (TaqMan assay no.)*
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	4333764F
Osteopontin	*SPP1*	Hs00959010_m1
C-X-C motif chemokine ligand 12	*CXCL12*	Hs03676656_mH
Vascular cell adhesion molecule 1	*VCAM1*	Hs01003372_m1
Angiopoietin 1	*ANGPT1*	Hs00181613_m1
Jagged1	*JAG1*	Hs00164982_m1
KIT Ligand	*KITLG*	Hs00241497_m1
Alkaline Phosphatase	*ALPL*	Hs01029144_m1
Osteocalcin	*BGLAP*	Hs00609452-g1
Type I collagen alpha 2 chain	*COL1A2*	Hs01028970_m1
Runt-related transcription factor 2	*RUNX2*	Hs00231692_m1
Osteonectin	*SPARC*	Hs00234160_m1
Aggrecan	*ACAN*	Hs00202971_m1
Distal-less homeobox 6	*DLX6*	Hs00231999_m1
SRY-box 9	*SOX9*	Hs00165814_m1
Runt-related transcription factor 3	*RUNX3*	Hs00231709_m1
Receptor activator of nuclear factor-kappaB ligand	*RANKL*	Hs00243519_m1
Osteoprotegerin	*OPG*	Hs00171068_m1
Lipoprotein Lipase	*LPL*	Hs00173425_m1
Osterix	*SP7*	Hs01866874_s1
Fatty Acid-Binding Protein 4	*FABP4*	Hs01086177_m1
Peroxisome proliferator activated receptor gamma	*PPARG*	Hs01115513_m1