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Elucidating the Acute Myeloid Leukemia-mediated Reshaping of Bone Marrow Stromal Niche

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CHAPTER 1: GENERAL INTRODUCTION

1 Bone Marrow Niche

Since its definition in 1978 by Schofield[1] bone marrow (BM) niche has aroused great interest. Firstly, because it is the microenvironment where hematopoietic stem cells (HSC) reside, maintain an undifferentiated state and self-renewal capacity or differentiate through the hematopoiesis up to mature blood cells[2]. Secondly, because the BM niche includes non-hematopoietic cell types and acellular components, such as extracellular matrix (ECM), chemical and physical factors, that tightly control HSC fate and consequently can be involved not only in normal hematopoiesis regulation but also in blood malignancies development [3–5].

Recently, the main insights about cellular identity, anatomical localization and specific functions of BM niche' components derive from landmark studies performed on murine BM using single-cells technologies [6–9]. An integrated analysis combining all the datasets in these studies defined fourteen meta-clusters of cell populations characterized by the expression of hematopoietic factors[10]. These clusters comprise endothelial cells (EC) (arterial, arteriolar and sinusoidal, 32% of all cells), mesenchymal stem and progenitor cells (adipogenic and osteogenic, 27%), osteoblasts (mature and immature, 5%), chondrocytes (16%), fibroblasts (18%), pericytes (1%), smooth muscle cells (0.1%), and Schwann cells (0.3%)[10]. Moreover, several i*n vivo* functional studies have described that skeletal and stromal cell populations, endothelial and other non-hematopoietic cells as well as differentiated hematopoietic elements are fundamental players of BM microenvironment regulation[11–15]. Nevertheless, the heterogeneity of the non-hematopoietic component of the human BM niche remains poorly defined, due to the extreme scarcity of stromal cells contained.

Initially, two distinct specialized niche milieus, composed of specific cells differently localized and that regulate different types of hematopoietic cells (endosteal and vascular niches) [16,17] were conceived in the BM. To date, this dichotomy has been overcome thanks to the improvement of technologies which have pave the way for important insights into the complexity of the human BM[11,18,19]. Indeed, these studies demonstrated that the endosteum is vascularized [20], and despite human HSC are found

predominantly located in perivascular regions of the BM[21,22], when HSC were transplanted in a murine model, they localize not only in trabecular areas of the BM[23] but also in direct contact with sinusoids, in particular with CD271^{+} BMSC[23,24], CXCL12-abundant reticular (CAR) cells or in presence of E-selectin microdomains on BM sinusoids[25]. These results allow to exceed the theory that more quiescence HSC reside in the endosteal niche[20] while the differentiative-HSC take place near to sinusoid[26–28], approaching to a new prospective of cooperation between the two niches. In conclusion, nowadays it is believed that the exchange between endosteal and vascular niches is continuous and that the HSC pool is in the balance between trafficking, proliferation and quiescence[29,30] (Fig.1).

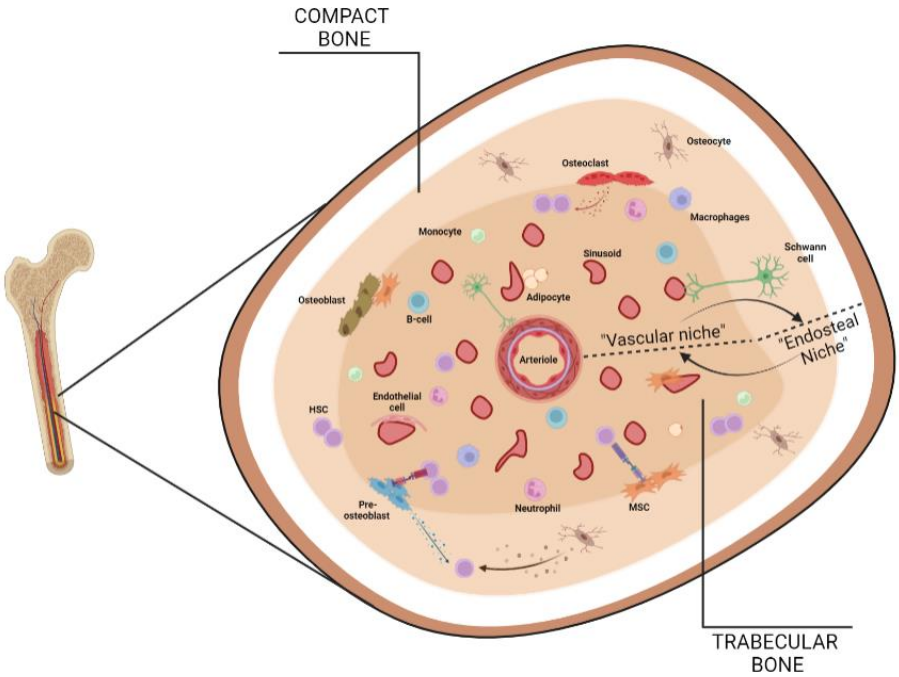


Fig.1 Bone marrow niche composition. *Figure created with Biorender.com*

1.1 Hematopoietic stem cells (HSCs)

HSCs are hematopoietic uncommitted progenitor cells, defined by their ability to undergo asymmetric cell division, allowing simultaneously self-renewal (generation of daughter cells identical to original HSCs) and multipotent hematopoietic

differentiation (generation of mature adult hematopoietic cell types)[31–34]. This feature places HSCs at the apex of hematopoiesis and enables them to sustain blood system homeostasis throughout the lifespan and to reconstitute the entire hematopoietic system following transplantation into an irradiated recipient[35]. Interestingly, this capacity provides the scientific basis for HSC transplantation (HSCT). However, self-renewal capability decreases from one transplant to another allowing to divide HSC into Long-term HSC (LT-HSC), the most primitive and less differentiated progenitors, and Short-term HSC (ST-HSC), which present a more limited self-renewal capability but differentiate in multipotent progenitors, which in turn give rise to all blood mature cells (Fig.2). Use of expanding cell surface marker panels and advanced functional analyses has revealed the presence of several phenotypically different HSC subsets with distinct self-renewal and repopulating capacities and biased toward selective lineage differentiation. Furthermore, single-cell analysis confirmed a big heterogeneity in HSC [36–38]. Moreover, the phenotypic definitions of the human HSCs are different from mouse[39–42].

HSCs' balance core functions such as quiescence, proliferation, and differentiation are controlled by autocrine stimuli and extrinsic cues from the niche[43]. In fact, all cell subsets composing the niche cooperate in the regulation of hematopoiesis through the secretion of soluble signals, including cytokines, hormones, growth factors, and through the expression of adhesion molecules and receptors[6,44].

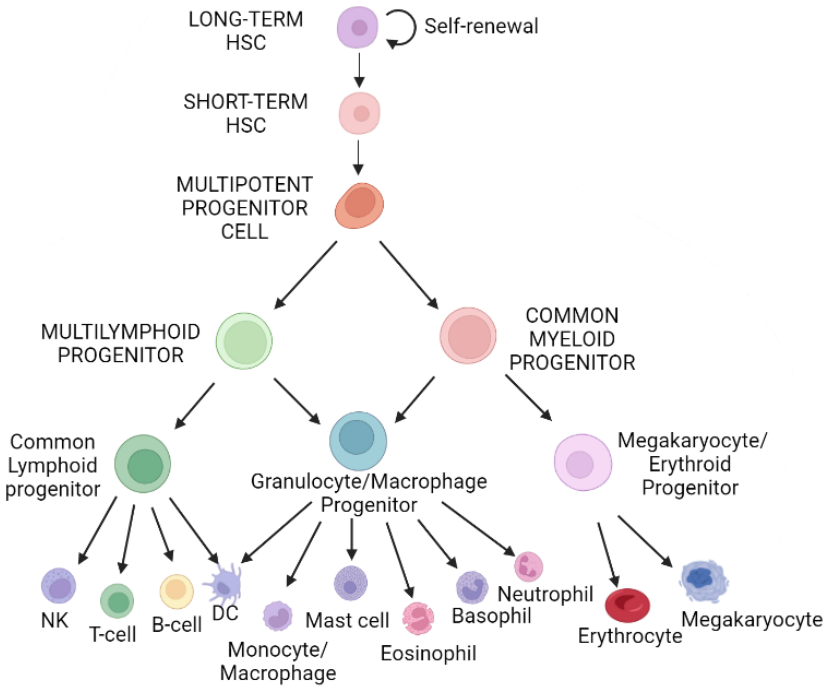


Fig.2 Hematopoiesis hierarchy. Adapted from Cheng et al[45] with *Biorender.com*

1.2 Bone marrow-derived Mesenchymal Stromal Cells (BMSCs)

BMSCs, also termed Mesenchymal Stromal Cells (MSCs), are a multipotent stromal cell population endowed with the capacity to differentiate into bone (osteoblasts), fat cells (adipocytes) and cartilage (chondrocytes), with a central role in HSC maintenance and hematopoiesis regulation, BM niche organization and skeletal tissues maintenance (Fig.3).

To date cultured BMSCs are characterized, as defined by the minimum criteria established by the International Society for Cellular and Gene Therapy (ISCT), by: (i) the ability to grow in adherence to plastic when cultured *in vitro*; (ii) the spindle-shaped morphology; (iii) the clonogenic potential (colony-forming unit-fibroblast; CFU-F); (iv) the expression of surface markers

CD105, CD90, CD73 and the lack of hematopoietic markers CD45, CD34, CD14/CD11b, CD79α, CD19 and human leukocyte antigen (HLA-DR); (v) the capability to differentiate *in vitro* into 3 mesodermal lineages[46].

An additional feature which makes BMSCs interesting is their immunoregulatory potential. Indeed BMSCs can modulate immune cells such as T, B and natural killer (NK) cells, monocytes and dendritic cells (DC), in response to surrounding pro- or anti-inflammatory stimuli, regulating the composition of BM immune microenvironment[47–50] (Fig.3).

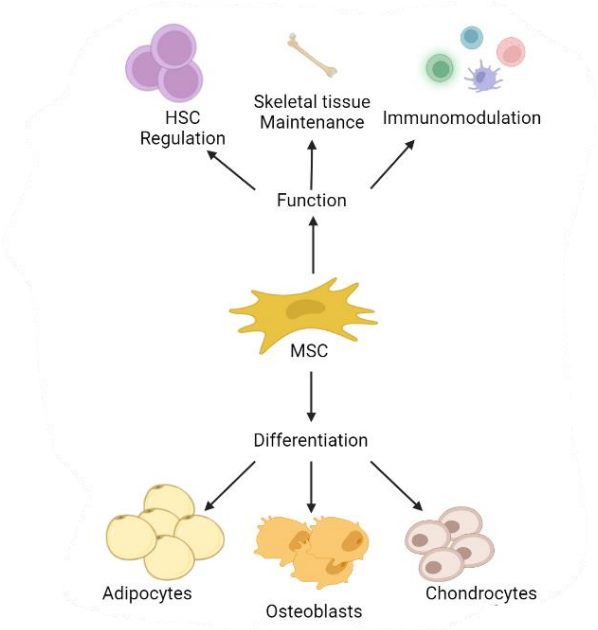


Fig.3 Mesenchymal stromal cells capabilities and functions. Figure created with Biorender.com

Detailed insight into the functional roles of primary stromal cells in regulating hematopoiesis[44] are provided by studies on murine models. These works use genetic approaches involving *in-vivo* labeling of cell types and ablation of candidate niche cells or niche factors such as leptin receptor (LEPR), Nestin (Nes), C-X-C motif ligand 12 (CXCL12), and neural/glial antigen 2 (NG2).

The widely represented BMSCs population in the perisinusoidal niche is characterized by high expression of CXCL12, SCF and

LEPR (CAR cells), has a high expression of adipogenic and osteogenic factors and the ability to differentiate into osteoblasts and adipocytes[51]. Ablation of CAR cells in mice led to a decrease in the HSCs population in the niche, associated with reduced production of CXCL12 and SCF [52]. Furthermore, BMSCs present a high cell-to-cell heterogeneity. Using single-cell RNA sequencing on murine BM stroma components, Tikhonova et al[8] and Baryawno[7] et al identified cell subtypes corresponding to multiple trajectories in BMSCs. Combined scRNA-seq and spatially resolved transcriptomics have revealed two different subsets of CAR cells: a more abundant Adipo-CAR cell population expressing adipocyte-related genes located near to sinusoids and a less abundant Osteo-CAR cell population enriched in osteolineage-related genes and located in avascular BM regions or associated with arteriole-like vessels[6].
Emerging studies have explored the single-cell transcriptome also of primary human BMSCs, providing the first important insights into their different identities and functions [19,53]. However, the low frequencies of BMSCs in human BM makes challenging investigate the heterogeneity of the BM stromal cell compartment even when employing single-cell approaches, limiting the analysis of human stromal cell functions to *in vitro* co-cultures. Indeed, compared to full mouse bone, human BM aspirates contain very few BMSCs, within the range of 0.001-0.01% of total cellularity. For this reason, approaches to increase BMSCs content from human material require enrichment applications by cell sorting strategies.
Recently, humanized models of the BM niche were employed as a new tool to study *in vivo* the human stroma in physiological and pathological contexts[54].

1.2.1 MSCA-1/TNAP and other markers of human primary BMSCs

Identification of human primary BMSCs has conventionally relied on prospective isolation usually by a combination of two or more surface markers. Reported markers for primary BMSCs include CD271 (LNGFR), CD106 (VCAM-1), STRO-1, CD146 (MCAM),

CD90 (THY-1), CD105 (Endoglin), frizzled-9, SSEA-4, CD51 (ITGaV), CD140A (PDGFRa), SUSD2 (W5C5), LEPR, CD203 (PrP), and MSCA-1/TNAP.

Mesenchymal stem cell antigen-1 (MSCA-1) or tissue-nonspecific alkaline phosphatase (TNAP) is expressed at the surface of primary BMSCs [55] and it is involved in a wide range of BMSCs features, from cell differentiation to immunomodulatory properties[56]. Among the main roles that TNAP can play, it turns out to be critical for the bone mineralization[57,58]. In fact, TNAP has the fundamental function to hydrolyze the extracellular pyrophosphate to inorganic phosphate, which combine with calcium[59] forming hydroxyapatite crystals, that once deposited generate new bone. Moreover, the inorganic phosphate generated by TNAP is transported into the cytoplasm through a sodium-coupled phosphate transporter (PiT-1), reaching the nucleus where it promotes gene expression of proteins involved in osteoblast differentiation[60], including TNAP itself. In fact, TNAP expression on the BMSCs surface significantly increases when BMSCs are under osteogenic stimuli. For this reason, we can afford to define TNAP as one of the first surface markers of osteogenic differentiation. Lastly, the main correlation between TNAP and osteogenesis is provided by the impaired mineralization showed by murine models presenting mutations in TNAP gene (*ALPL*)[61] (Fig.4).

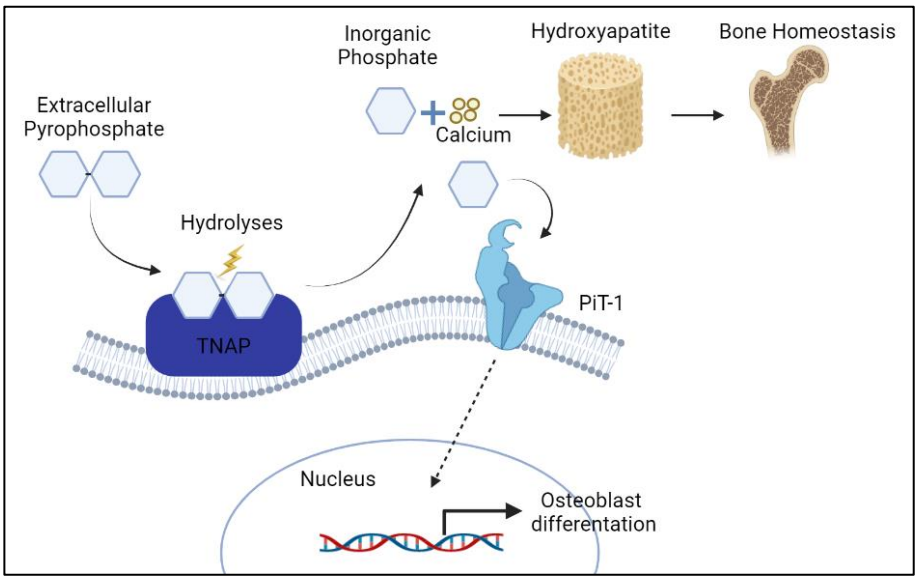


Fig.4 Schematic representation of the TNAP activities. *Figure created by Biorender.com*

1.2.2 Hematopoietic support mechanisms of BMSCs

BMSCs exert their function through direct interaction with HSCs and by secreting several paracrine factors.
BMSCs express adhesion molecule as N-Cadherin which favor the regulation of hematopoietic stem and progenitor cells (HSPCs) in BM niche[62]. Moreover, the BMSCs release of supportive factors is the principal molecular mechanism regulating HSC homeostasis *in vivo* and in BMSC-based co-culture systems. Among the many components of BMSCs secretome, CXCL12 (C-X-C Motif Chemokine Ligand 12), stem cell factor (SCF) and Kit ligand (Kitl) play a pivotal role in HSC maintenance. Other BMSC-secreted factors were observed to exert important roles in inflammatory reactions, cell homing, and regulation of apoptosis, fibrosis and angiogenesis, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiopoietin (ANGPT), and hepatocyte growth factor (HGF) (Table 1)[63,64]. Another method through which BMSCs interact, modify, and respond to the surrounding environment is by releasing extracellular vesicles and exosomes.

In vitro, BMSC-derived extracellular vesicles have been shown to promote the proliferation of HSC by triggering the WNT/b-catenin pathway[65].

Secreted Factors	Function
Basic fibroblast growth factor (bFGF)	Cell Survival, Proliferation and Differentation
Cell survival, proliferation, and differentiation Insulin-like growth factor (IGF)	
Secreted frizzled-related protein-1 (SFRP1)	
Secreted frizzled-related protein-2 (SFRP2)	
Stanniocalcin-1 (STC-1)	
Transforming growth factor β (TGF-β)	
miR-10b-5p, miR-22-3p, miR-191, miR-222, miR-21, let-7a	
Metalloproteinase-1 (MMP1)	Remodeling of extracellular matrix
Remodeling of extracellular matrix Metalloproteinase 9 (MMP9)	
Plasminogen activator (PA)	
Tumor necrosis factor-α (TNF-α)	
Angiopoietins (ANGs)	Angiogenesis
Angiogenesis Fibroblast growth factor-2 (FGF-2)	
Transforming growth factor β (TGF-β)	
Vascular endothelial growth factor (VEGF)	
miR-132 [55] miR-222, miR-21, let-7f	
Hepatocyte growth factor (HGF)	Immunomodulatory
Immunomodulatory Human leukocyte antigen G5 (HLA-G5)	
Indoleamine 2,3-dioxygenase (IDO)	
Inducible nitric oxide synthase (iNOS)	
Interleukin-6 (IL-6) [61] Interleukin-10 (IL-10)	
Leukemia inhibitory factor (LIF)	
Prostaglandin E2 (PGE2)	
Transforming growth factor β (TGF-β)	
miR-143-3p	

Table 1 Endocrine factors secreted by bone marrow mesenchymal stem cells (adapted from Chu et al [63])

Lastly, BMSC express Notch ligands, including Jagged-1, Jagged-2, DLL-1, and DLL-4, which are responsible for the activation of Notch signaling in HSCs, a key pathway controlling HSC growth and differentiation during development [66].
Notch signaling is a pivotal pathway involved in cellular proliferation, differentiation, and apoptosis[67]. It is composed by 4

receptors (Notch-1/2/3/4) and 5 Delta-like ligands (Dll-1/3/4, Jagged-1/2)[68]. Notch receptors are in turn composed of 3 domains: extracellular, intracellular, and transmembrane. Once the ligand binds the extracellular domain of Notch receptor, an extracellular and an intracellular proteolytic cut, performed by metallopeptidase and γ-secretase respectively, release into cytoplasm the intracellular domain (NICD) which is subsequently transported to the nucleus. Here, NICD forms a complex with several transcription regulators (such as CSL, CBF-1, Lag-2, MAML-1, and P300/CβP), inducing the expression of downstream Notch transcriptional factors, especially Hey-1 (Hairy/enhancer-of-split related with YRPW motif 1) and Hes-1 (hairy and enhancer of split1) (Fig.5).

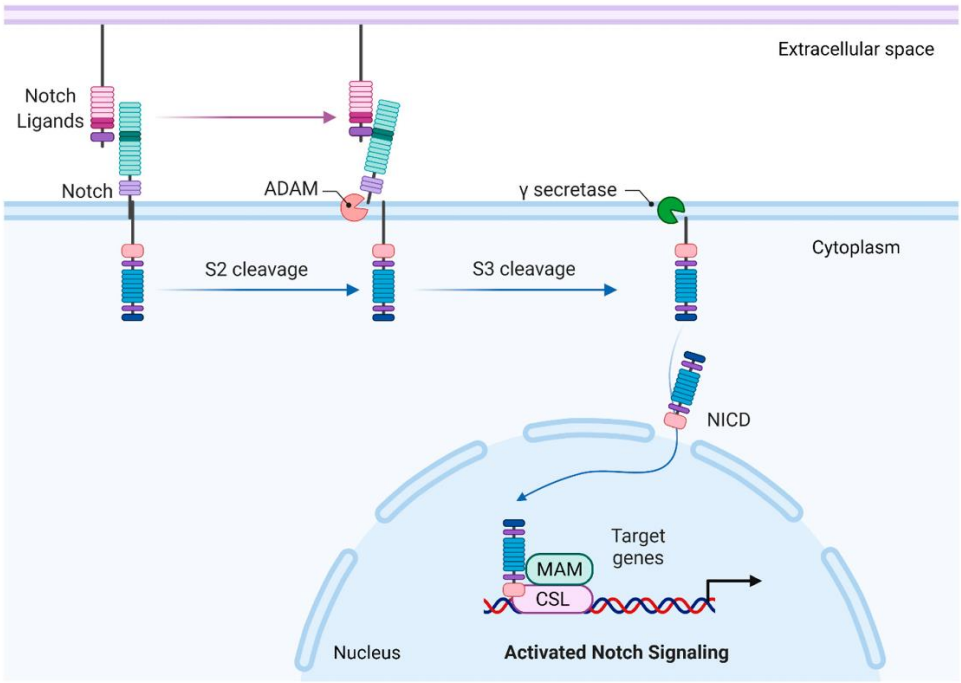


Fig.5 A simplified view of canonical Notch signaling [69]

Notch signaling plays a pivotal role in hematopoiesis. In fact, Kadekar and colleagues showed that an higher expression of β-catenin, DLL-1, Jagged1, Hes1, Notch1, and NICD in BMSCs prevent the apoptosis of primitive HSCs[70]. While Duryagina and

colleague observed that Jagged1 expression by BMSCs induces the release of CXCL12, thus supporting proliferation, migration, and adhesion of $CD34^+$ progenitors[71]. Moreover, co-culture of BMSCs and HSCs trigger Notch signaling in both cell type, regulating HSCs proliferation[72]. Interestingly, Notch signaling favors osteoblast maturation and controls BMSCs, maintaining them in an undifferentiated state[73,74].

1.3 Osteoblastic cells

Besides BMSCs, their derivatives osteoblastic cells, including osteoprogenitors, pre- and mature osteoblasts, and osteoclasts, are located in the region lining the inner bone surface[27] and are responsible for the dynamical balance of bone formation. Moreover, osteoblastic cells are the primary contributors of the endosteal niche and were among the first cells to be identified as regulators of hematopoiesis. Indeed, osteoblasts synthesize many factors affecting HSC proliferation and their more differentiated progeny maturation (erythroid differentiation), such as osteopontin (OPN) and erythropoietin (EPO)[75,76]. Moreover, they regulate HSC self-renewal through specific pathways, such as Notch signaling. In addition, osteoblasts express adhesion molecules and produce different cytokines and growth factors which are known to be closely related to HSCs homing, quiescence and mobilization, such as N-cadherin, vascular cell adhesion molecule 1 (VCAM-1), CXCL12[77], granulocyte colony-stimulating factor (G-CSF)[78], ANGPT-1[79], and thrombopoietin (TPO)[80]. Besides osteoblasts, their differentiated form, osteocytes, further contribute to the control of HSCs through the secretion of G-CSF[81] (Fig.6).

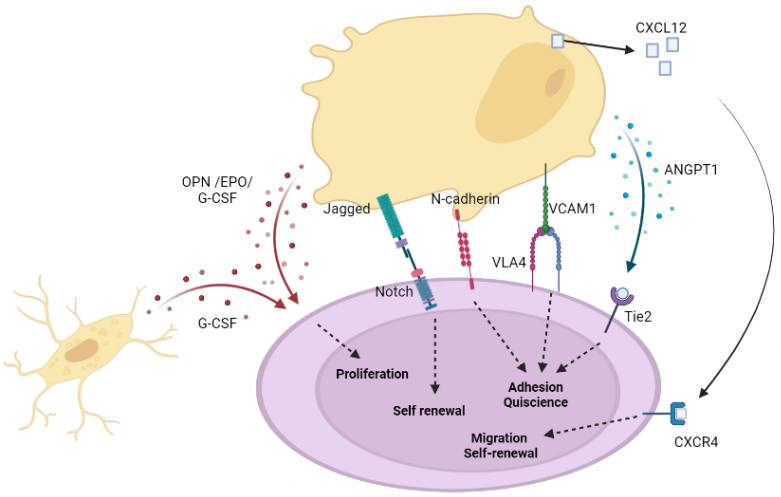


Fig 6. Schematic overview of the interactions between osteoblasts and hematopoietic stem cells (HSCs) (adapted from Bourdieu et al[82] with *Biorender.com*).

In vivo studies have extensively investigated the role of osteoblasts in the support of hematopoiesis. Two landmark studies showed that transgenic animals with increased osteoblast activity through stimulation of the parathyroid hormone (PTH)-related protein receptor (PPR) or inactivation of the bone morphogenic protein receptor-1a (BMPR-1a), exhibited an increase in the number of BM HSCs[83,84]. Despite this evidence, several subsequent studies reconsidered the role of osteoblasts in the maintenance of HSCs. Visnjic and colleagues reported that conditional ablation of osteoblasts did not induce any severe loss in BM HSCs pool size, but rather promotes the loss of more differentiated cells, especially B cell progenitors[85]. Furthermore, a study using strontium as a treatment to expand osteoblasts, showed that it was not correlated with HSC increase[86]. Similarly, Calvi et al showed that PPR-dependent expansion of osteocytes and mature osteoblasts did not increase BM HSCs[87]. In particular, conditional deletion of HSC regulators CXCL12 and SCF in osteoblasts did not significantly affect HSCs[12,88].

However, more recent studies reattracted interest to osteoblastic cells by demonstrating that dormant HSCs that are resistant to myeloablation were found to be in contact with N-cadherin^{+} osteoblastic precursors in the endosteal region[89]. Furthermore, ablation of N-cadherin+ cells or inactivation of the SCF gene in them negatively affected HSCs maintenance during homeostasis and regeneration. In a recent *in vitro* co-culture study, adherence to osteoblasts favored self-renewal of HSCs[90].
These latest findings have also suggested that the degree of involvement in hematopoiesis depends on osteoblast state of differentiation[91]. Raaijmakers and colleagues proved that genetic modifications on osteolineage precursors affect HSC proliferation, while same alterations in mature OB have no effects on HSCs[4].

1.4 Niche modeling

As the study of BMSCs and osteoblast contribution to hematopoiesis *in vivo* is hindered in humans, reliable models of the human BM niche are fundamental for the confirmation of findings arising from murine models.
To date, *in vitro* 2D systems are still the primary approach used to study hematopoiesis and the crosstalk between HSCs and BM niche components. The first and most widely used, is the *in vitro* co-culture model based on Dexter system[92], in which HSCs are seeded on an established feeder stromal cell layer (BMSCs, macrophages, endothelial cells, adipocytes, fibroblasts) in a controlled environment. This 2D niche model allows the direct cell manipulation and the control of experimental variables. It provided countless information regarding interactions and co-dependency between HSCs and stromal cells, especially about the primary role of BMSCs and osteoblasts to HSCs support through the secretion of soluble factors [78,93]. However, it fails at mimicking the complexity of the BM microenvironment. To overcome this issue, different 3D scaffold have been developed using inorganic (hydroxyapatite, tricalcium phosphate), natural (collagen, fibrin, heparin) or synthetic polymers (polyurethane,

poly-L-lactic acid, polyethylene glycol)[94]. These scaffolds give support for cell proliferation and a controlled cell interactions due to its spatial organization. To integrate further BM niche' key factors, as regulated soluble factor concentration, oxygen gradient and mechanical stress applied by blood flow, it was developed a "4D system" which combine 3D organoids with perfusion-based bioreactor systems[95]. 3D scaffold-based microfluidic chips have been introduced for the generation of a "BM-on-a-chip"[96–98]. These complex systems allow to maintain the cell spatial positions and the correct perfusion as in *in vivo* BM niche, providing more accurate recapitulation of human physiology and pathophysiology, in addition to making an important contribution to assess pharmaceutical treatments [99–101] (Fig.7).

Moving on *in vivo* systems, xenograft models in which human cells engrafted on the BM niche of mice are used. The importance of developing a humanized BM niche model has been highlighted by the fundamental findings obtained by immunosuppressed and genetically modified mice expressing human growth factors. To date the great advancement reached in humanized mouse model is the use of human BM niche-forming cells to generate human-mouse chimeric BM tissues, which can provide pivotal information on the interactions between human stroma and HSCs. Various protocols for the generation of humanized BM models have been developed. All approaches are based on an *in vitro* step to prepare an implantable structure with BMSCs and a cell carrier material[22,102–105] but some protocols add an *in vitro* cell differentiation step like the co-seeding of human endothelial cells or the addition of osteogenic factors before implantation in mice to ameliorate the *in vivo* differentiation. At this point, the implantable structures are transplanted subcutaneously in the back of immunodeficient mice for the humanized niches *in vivo* formation. Human hematopoietic cells can be integrated in the system at different steps[54]. The generated-ossicles present both myelo-supportive marrow stroma and hematopoietic tissues and its progeny of human origin. Sinusoidal endothelium, nerve

fibers, residual hematopoietic cells and osteoclasts are derivatives of mouse host[106].

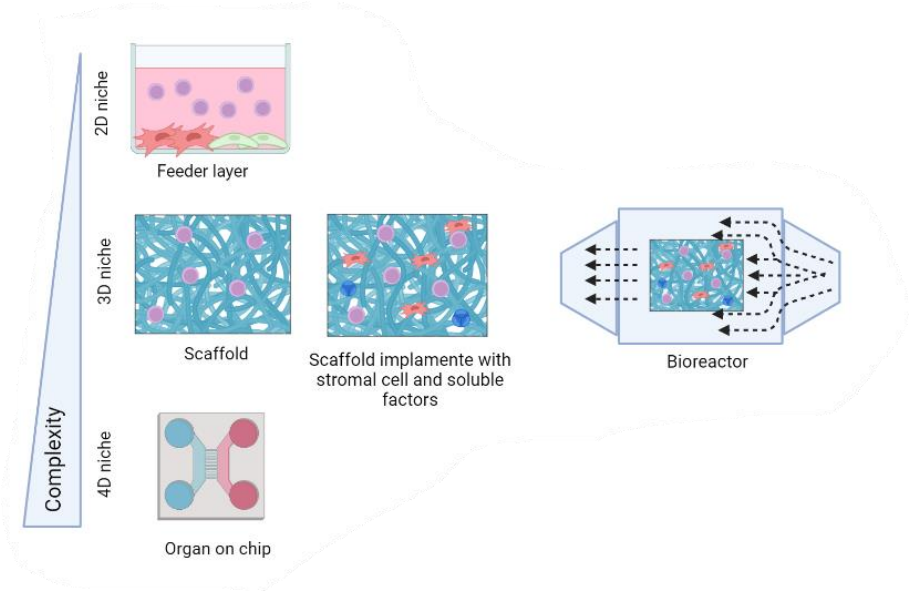


Fig.7 Schematic drawing of the development of *in vitro* BM niche models (Figure adapted from Xiao et al [107] with *Biorender.com*)

1.5 Niche transformation in leukemia

Numerous data demonstrated a direct and indirect involvement of the BM niche in supporting leukemic cells during the development of malignancies. Malignant cells, in addition to perturbation of normal hematopoiesis, can substantially modify the BM niche components, which undergo modifications that facilitate disease progression. These leukemogenic features include changes associated with increased hypoxia, angiogenesis and inflammation, advantageous release of pro-survival factors, competition in niche space with healthy HSCs, stromal reprogramming and physical protection against chemotherapy[108,109].

Particularly, BMSCs can play a pivotal role in the generation of a leukemia-supportive microenvironment, influencing the disease pathogenesis at different steps including onset, maintenance and progression. Leukemia cells hijack BMSCs, which in turn protect them from chemotherapeutic drugs by several mechanism such as cytokines, pro-survival stimuli and exchanging organelles.
Furthermore, it has been widely described in murine models that genetic lesions of cells in the niche can have a disruptive effect on hematopoiesis while promoting hematologic disorders.
In the next chapter regarding AML, we will deepen the specific features of the BM niche during the development of myeloid malignancies.

2 Acute Myeloid Leukemia

2.1 Definition, Incidence and Diagnosis

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder characterized by rapid growth and accumulation of myeloid immature precursors, named myeloblasts, in lymphopoietic organs such as BM, peripheral blood (PB), spleen, and lymph nodes[110,111]. The primary effect of their accumulation is the BM failure and the associated alteration of normal hematopoiesis[111] that in turn induce the onset of the typical symptoms of AML: anemia, neutropenia, higher susceptibility to infections, angina and chest pain[110,112]. If untreated, infections or hemorrhages can cause the death of patients. These circumstances make the readily and proper diagnosis paramount for AML patients' survival.
AML is the most diagnosed leukemia in adults and represent the 15-20% of cases of leukemia in children[111]. It affects mainly adult men [113,114] and its incidence increases with age from 1.3 cases per 100,000 for those under 65 years to 12.2 cases per 100,000 for those over 65 years [111]. Relative survival for adults is 34% at 1 year from the onset and 27% at 5 years, values that decrease with increasing of the patient age. The 5-year survival rate for children younger than 20 years is almost 69%[115].

Multiple tests must be performed for a correct AML diagnosis, due to the extreme heterogeneity of the disease (Fig.8). For this reason, at the AML onset, BM aspirate, PB and cerebrospinal fluid samples are collected and analyzed for the presence of blasts. After the confirmation of the blast presence, it is necessary a morphological analysis to identify their myeloid lineage origin. Blood and marrow smears are morphologically examined after staining with May-Grunwald-Giemsa or Wright Giemsa. For diagnosis of AML, it is required a blast count of 20% or more. For cases presenting with less than 20% blasts (but more than 10%), the identification of recurrent genetic abnormalities that define specific AML subtypes (see Classification) is now considered to establish the diagnosis. To clarify thew blast maturation level, cytochemical stains were used, such as Sudan-Black-B (positive stain of granulocytic cells and eosinophils; weak monocytic staining) and myeloperoxidase staining (positive stain of myeloperoxidase presents in the primary granules of myeloid cells as azurophils granules, neutrophils and monocytes. Early myeloblasts are negative, with granular positivity appearing progressively as they mature; Auer rods are strongly MPO positive).

Immunophenotyping by multiparameter flow cytometry is required for a more precise patient-specific diagnosis by identifying cell surface and intracellular markers of AML blasts. Because of the heterogeneity of marker expression on AML blasts, the identification of patient-specific leukemia-associated immunophenotype (LAIP) is important for subsequent minimal residual disease (MRD) monitoring by flow cytometry. Some of the main markers detected are CD33, CD13, MPO, HLA-DR, CD34 and CD117[112,116].

Cytogenetic analysis (chromosome banding or fluorescence in situ hybridization) is mandatory for the detection of specific abnormalities like PML::RARA, RUNX1::RUNX1T1, CBFB::MYH11, KMT2A (MLL), and MECOM (EVI1) gene fusions, or myelodysplasia-related chromosome aberrations[112,116], such as loss of chromosome 5q, 7q, and 17p material.

Molecular testing should screen for all the genetic abnormalities that define disease and risk categories or that present targeted treatments. These tests can be performed by commercially available gene panel diagnostics, which simultaneously test mutations and rearrangements, paying attention to false-negative results due to hematopoietic somatic reversion typical in myeloproliferative disease. When AML with germline predisposition is suspected a dedicated gene panel including known predisposing alleles should be used.

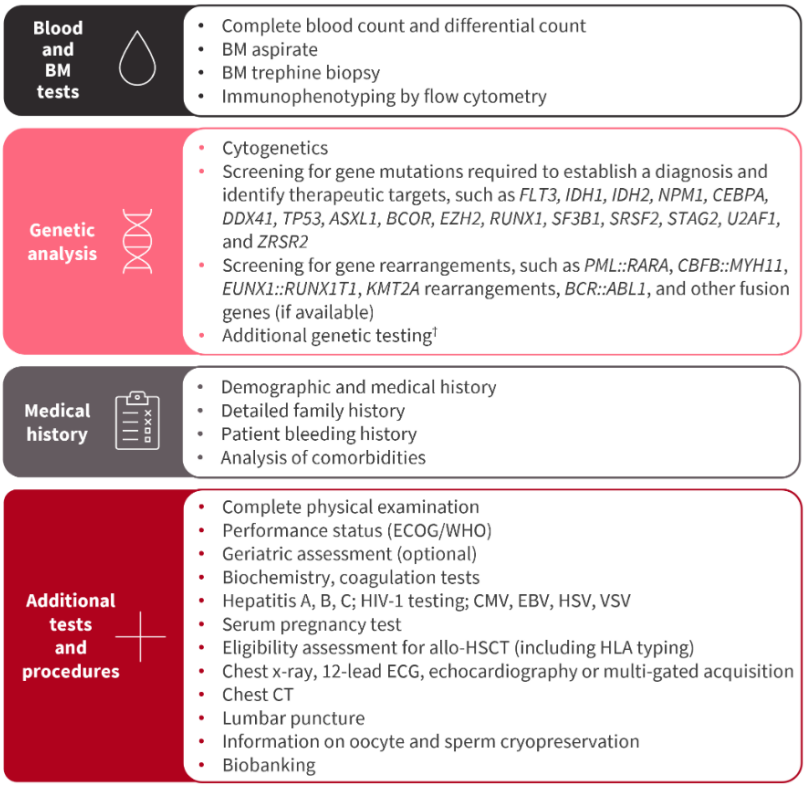


Fig.8 Tests and procedures at diagnosis for a patient with AML [117]

2.2 General Classification

Because AML is extremely heterogeneous, its classification results challenging. In the 1970s, AML was classified according to the French-American-British (FAB) classification system in

which it was subdivided into 8 subtypes (M0-M7) based on morphological and immunephenotype/cytochemical characteristics of the leukemia cells[112] (Fig.9).

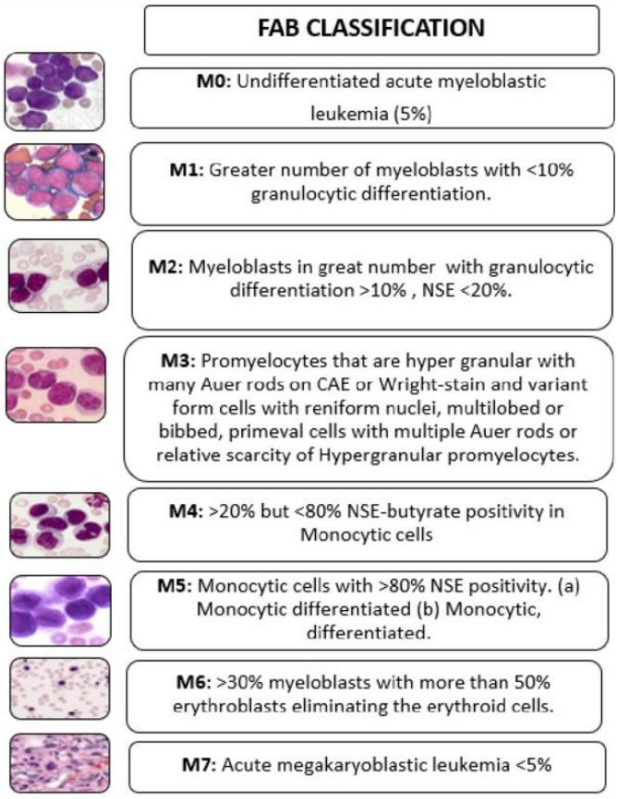
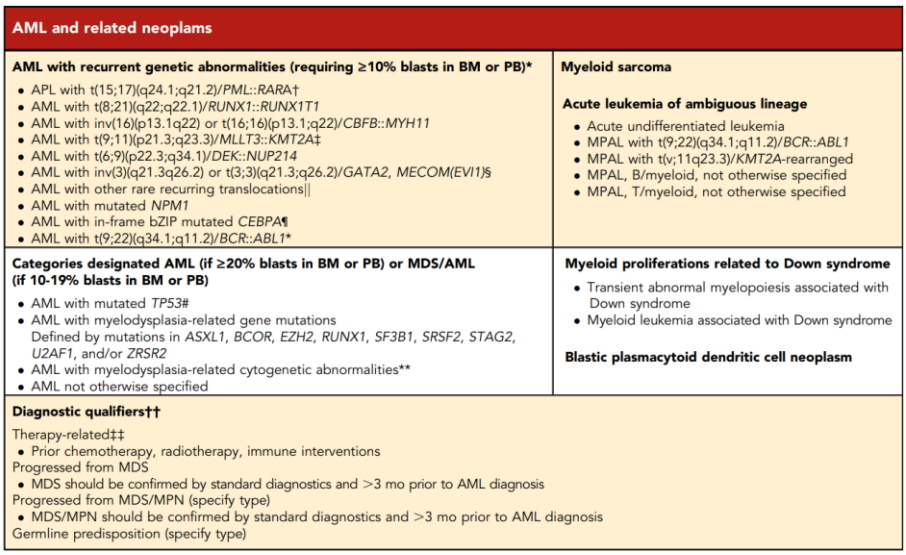


Fig.9: French-American-British (FAB) classification of AML[118]

In 2001 the World Health Organization (WHO) established a new classification system[119], which was following revised in 2008. This system, according to morphology, immunophenotype, cytogenetic and mutational profiles, and clinical aspects, subdivides AML and related neoplasms into 7 major subgroups: AML with recurrent genetic abnormalities, AML with MDS-related changes, therapy-related myeloid neoplasms, AML not otherwise specified, myeloid sarcoma, myeloid proliferation related to Down syndrome, and blastic plasmacytoid dendritic cell neoplasm[112,116] (Fig.10). Genetic aberrations are given priority in

defining AML disease classification because of their strong impact on disease outcome.



AML and related neoplams

AML with recurrent genetic abnormalities (requiring ≥10% blasts in BM or PB)*

- APL with t(15;17)(q24.1;q21.2)/*PML::RARA*†
- AML with t(8;21)(q22;q22.1)/*RUNX1::RUNX1T1*
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB::MYH11*
- AML with t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A*‡
- AML with t(6;9)(p22.3;q34.1)/*DEK::NUP214*
- AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2, MECOM(EVI1)*§
- AML with other rare recurring translocations||
- AML with mutated *NPM1*
- AML with in-frame bZIP mutated *CEBPA*¶
- AML with t(9;22)(q34.1;q11.2)/*BCR::ABL1**

Myeloid sarcoma

Acute leukemia of ambiguous lineage

- Acute undifferentiated leukemia
- MPAL with t(9;22)(q34.1;q11.2)/*BCR::ABL1*
- MPAL with t(v;11q23.3)/*KMT2A*-rearranged
- MPAL, B/myeloid, not otherwise specified
- MPAL, T/myeloid, not otherwise specified

Categories designated AML (if ≥20% blasts in BM or PB) or MDS/AML (if 10-19% blasts in BM or PB)

- AML with mutated *TP53*#
- AML with myelodysplasia-related gene mutations
  Defined by mutations in *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, and/or *ZRSR2*
- AML with myelodysplasia-related cytogenetic abnormalities**
- AML not otherwise specified

Myeloid proliferations related to Down syndrome

- Transient abnormal myelopoiesis associated with Down syndrome
- Myeloid leukemia associated with Down syndrome

Blastic plasmacytoid dendritic cell neoplasm

Diagnostic qualifiers††

Therapy-related‡‡
- Prior chemotherapy, radiotherapy, immune interventions

Progressed from MDS
- MDS should be confirmed by standard diagnostics and >3 mo prior to AML diagnosis

Progressed from MDS/MPN (specify type)
- MDS/MPN should be confirmed by standard diagnostics and >3 mo prior to AML diagnosis

Germline predisposition (specify type)

Fig.10 WHO revised AML and related neoplasm classification[116]

Of greater clinical relevance is the classification of AML into 3 prognostic risk groups (favorable, intermediate, and adverse), relying on cytogenetics and known molecular abnormalities at initial diagnosis[120]. This classification, proposed by The European LeukemiaNet (ELN) in 2017, is a fundamental tool for outcome prediction in AML. It has recently been updated (2022 ELN) (Fig.11). Now, in addition to baseline genetic characterization, the importance of response to initial therapy and assessment of early MRD in individual risk assignment were highlighted.

Risk category†	Genetic abnormality
Favorable	• t(8;21)(q22;q22.1)/*RUNX1*::*RUNX1T1*†,‡ • inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB*::*MYH11*†,‡ • Mutated *NPM1*†,§ without *FLT3*-ITD • bZIP in-frame mutated *CEBPA*‖
Intermediate	• Mutated *NPM1*†,§ with *FLT3*-ITD • Wild-type *NPM1* with *FLT3*-ITD (without adverse-risk genetic lesions) • t(9;11)(p21.3;q23.3)/*MLLT3*::*KMT2A*†,¶ • Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23.3;q34.1)/*DEK*::*NUP214* • t(v;11q23.3)/*KMT2A*-rearranged# • t(9;22)(q34.1;q11.2)/*BCR*::*ABL1* • t(8;16)(p11.2;p13.3)/*KAT6A*::*CREBBP* • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2, MECOM(EVI1)* • t(3q26.2;v)/*MECOM*(*EVI1*)-rearranged • −5 or del(5q); −7; −17/abn(17p) • Complex karyotype,** monosomal karyotype†† • Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, and/or ZRSR2*‡‡ • Mutated *TP53*[a]

Fig.11 ELN-2022 risk stratification of AML by genetic abnormalities at diagnosis[116]

2.3 Pathogenesis

The onset of AML can be traced back to several causes. First of all, the exposure to ionizing radiation, benzene and chemotherapeutic agents combined with alterations in detoxifying enzymes (therapy-related AML)[113]. AML can also develop from myelodysplasia (MDS) or chronic myeloproliferative disorders. Less often, AML arises in patients presenting predisposing germline mutations in genes, such as ANKRD26, CEBPA, DDX41, ETV6, GATA2, RUNX1, SRP72, TERC, TERT or TP53[121]. Finally, congenital syndromes, such as Down syndrome, are associated with higher risk of AML development. However, the higher percentage of AML cases arises de novo[112]. AML develops from the serial acquisition of somatic mutations in HSC and progenitor cells with the capacity to self-renew and propagate the neoplastic clone.

2.4 Molecular signature

While approximately 50% of AML cases have a normal karyotype[122], the remaining 50% demonstrate an abnormal karyotype with multiple abnormalities [123,124], such as trisomy 8 and 21, monosomy 5 and 7, and various rearrangements such as t(8;21), t(15;17), and inv(16)[125,126]. For AML cases cytogenetically normal, specific gene mutations and/or changes in gene expression were identified and are now used to predict outcomes and help guide treatment for AML patients. Eight functional categories of genes that are commonly mutated in de novo AML have been described: signaling genes (FLT3, KRAS, NRAS and KIT mutations); epigenetic homeostasis genes with 2 subcategories, chromatin-modifying genes (ASXL1 and EZH2 mutations, MLL fusions) and methylation-related genes (DNMT3A, TET2, IDH1, and IDH2 mutations); nucleophosmin gene (NPM1 mutations); spliceosome-complex genes (SRSF2, SF3B1, U2AF1, and ZRSR2 mutations); cohesin-complex genes (RAD21, STAG1, STAG2, SMC1A, SMC3 mutations), myeloid transcription factors (RUNX1, CEBPA, and GATA2 mutations, RUNX1-RUNX1T1, PML-RARA, CBFB-MYH1 fusions); and tumor suppressor genes (WT1, TP53 mutations with PTEN and DMM2 deregulations). Most cases of AML present clonal heterogeneity at the time of diagnosis. Driver mutations, such as DNMT3A, TET2, and ASXL1, appear early in AML clones and represent a common pre-malignant state that increases in prevalence with age. These mutations preexist in the background before HSPC acquire the initiating mutations (NPM1, DNMT3A, or IDH1) leading to AML pathogenesis. Only a limited number of gene mutations have been assigned the role of triggering factor in AML pathogenesis while additional cooperative mutations are required to generate the founding malignant clone. Similarly, clonal evolution is also detected when relapsing. During treatment, AML cells acquire a small number of additional cooperating mutations from the primary clone that contribute to disease progression, resulting in resistance to chemotherapy and playing an important role in relapses. Notably, more than

25% of AML patients carry no mutations in the known leukemia-associated genes[127].

2.5 Leukemia stem cells (LSCs)

AML cell populations are organized in a hierarchical structure dominated by a rare and heterogeneous subset of cells called leukemic stem cells (LSCs). LSCs are capable of initiating and maintaining the disease and have been shown to be responsible for disease relapse due to their chemoresistance properties [111,128,129]. LSCs are found mainly in $CD34^+CD38^-$, but also in $CD34^+CD38^+$ and, to a lesser extent, $CD34^-$ fractions[130,131]. Due to the central role of LSCs in AML pathogenesis and progression, their therapeutic targeting and elimination are mandatory to improve the outcome. LSCs properties originate from mutations occurring in HSCs or more differentiated progenitors. These mutations up-regulate survival pathways (NF-κB, Wnt/β-Catenin, PI3K/Akt/mTOR, JAK/STAT)[132–135], stemness genes (MLL, VEGFB, JAG2, IGF1R) [136] and anti-apoptotic genes (BCL2 BCL2L1)[111,137–139], while down-regulate genes involved in DNA repair and tumor suppression (PTEN). Further alterations can involve Wnt/B-catenin, Hedgehog and Notch signaling and levels of microRNA (miR-9 and miR-126)[133,140,141].
Dick and colleagues demonstrated that LSCs and normal HSCs share several features, including the apex of leukemic/normal hematopoiesis hierarchy, spatial and functional distribution[142,143], quiescence, pluripotency, self-renewal capacity[128,129,144] and repopulating potential when transplanted into immunodeficient mice[132]. Moreover, the quiescent state and the higher expression of multidrug efflux pumps increase the resistance of LSCs to chemotherapeutic drugs. Furthermore, HSCs and LSCs exhibit similar phenotype ($CD34^+CD38^-CD71^-$HLA-DR^-)[111] making the discrimination between the two cell type challenging. However, LSCs express also specific antigens[137,145–148] (Fig.12) and present a distinctive molecular and metabolic profile, that could be exploited for selective targeting. The application of single-cell technologies in the AML context will help in the identification of novel LSC-specific targets.

Marker	Identified as	Expression			
		Normal	In AML (%)	HSC	CD34+ CD38− LSC
IL1RAP	IL1R3	T cells	79	−	+
CLL-1	CLEC12A, MICL, DCAL-2	Myeloid cells	70	−	+
TIM-3	T-cell Ig Mucin 3	Activated T cells, NK cells	91	−	+
CD2	SRBC, LFA2, T11	T cells, NK cells	87	−	+
CD7	GP40, TP41, LEU-9	T cells	43	−	+
CD11b	Integrin alpha M, Mac-1	Myeloid cells	55	−	+
CD22	BL-CAM, Siglec-2	B cells	51	−	+
CD25	IL2RA, TAC	Activated B and T cells	25	−	+
CD33	P67, Siglec-3	Myeloid cells, NK cells	82	+	++
CD44	Adhesion molecule	Ubiquitously	100	+	++
CD45RA	Tyrosine phosphatase receptor type C	T cells, myeloid cells	65	−	+
CD47	Integrin-associated protein (IAP)	Ubiquitously	100	+	++
CD56	N-CAM, MSK39	NK cells, activated T cells	32	−	+
CD96	TACTILE	Activated T cells	33	−	+
CD99	MIC2, single-chain type-1 glycoprotein	Myeloid cells	83	−	+
CD123	IL3R	Myeloid cells	82	+	++

Fig.12 Leukemic stem cell markers[149]

2.6 Treatments

The gold standard treatment of AML patients involves an initial induction phase followed by a consolidation phase. The first one, colloquially called the 7+3 regimen, is characterized by 7 days of continuous infusion of cytarabine (100mg/m^2/daily) and 3 days of anthracycline (60-90mg/m^2 of daunorubicin or 10-12 mg/m^2 of idarubicin). The aim of the induction phase is to reduce the leukemic bulk favoring the achievement of complete remission (CR)[150]. This goal is reached in up to 80% of patients with favorable risk disease and 50-60% of those with intermediate/adverse risk disease[120,151,152]. Several trials have now shown that higher dose of anthracycline (90 versus 45 mg/m^2) in both younger and adults results in higher CR rates and increases the duration of overall survival[151,152]. Furthermore, the outcomes have recently improved with the addition to the traditional 7+3 induction chemotherapy of various targeted drugs, such as midostaurin for patients with FLT3 mutation[153] or Gemtuzumab ozogamycin for patients CD33 positive[154] with favorable and intermediate-risk disease or Venetoclax, a

selective inhibitor of the anti-apoptotic protein BCL2, for patients with adverse risk disease[155].

The consolidation phase is pivotal to extending CR, eradicating the MRD and preventing relapse[112,120]. The type and duration of this phase depend on risk group classification[156]. Favorable and intermediate risk patients are usually treated with intensive chemotherapy (2-4 cycles of intermediate-dose cytarabine), with or without targeted therapies[120]. Instead high risk or resistant to primary treatment patients are prepared for allogeneic HSCT, following previous evaluation of transplant eligibility and comorbidity index[157].The absolute decrease in the risk of disease relapse must be more than the risk of non-relapse mortality to justify allogeneic HSCT as post-remission therapy. All patients with the adverse risk profile and most of intermediate risk profile meet these criteria. HSCT is usually recommended when the relapse incidence is expected to be 35-40%. Nowadays, a higher number of AML patients can benefit from HSCT thanks to the increased use of mismatched and unrelated donors. Moreover, no-myeloablative or reduced-intensity conditioning regimens have opened the possibility to treat also elderly patients (>75 years old). Despite the advances in patients' stratification and the several available treatment options, 5-years survival of AML pediatric patients remains around 70%[158], while 30% relapse in less than 1 year. Instead, elderly patients present an 80% of probability to relapse and their 5-years survival is lower, due to their sensibility to disease complications and/or the side effects of the treatment[113,121,158].

2.6.1 New therapies

For much of the 20th and early 21st century treatment paradigms were unchanged with survival curves remaining constant for many decades. Recent advances in our understanding of the genetic variations in the disease have led to some promising new genomically defined targeted therapies with the hope for improved survival and less toxic treatment. Some of these therapies like FLT3 inhibitors and Isocitrate Dehydrogenase (IDH) 1 and 2 inhibitors have been tested in clinical trials and are

now approved for patients who harbor these mutations. There are other targeted agents directed against various mutations seen in AML that are currently been investigated in clinical trials (e.g., selinoxor, a novel inhibitor of the nuclear exporter CRM1). Parallel to novel targeted therapy, immunotherapy is another promising approach to AML treatment that includes multiple immune modulating strategies, such as checkpoint inhibitors, antibody-drug conjugates, bispecific T-cell engager antibodies, and chimeric antigen receptor (CAR)-T/NK cell therapy. Immunotherapy is an appealing perspective because of the theoretical potential to specifically target malignant cells while sparing healthy tissues and minimizing the off-target effects typical of conventional chemotherapy. In this context, the identification of novel antigens is mandatory. In fact, the success of this type of treatment hinges on appropriate target selection. Finally, of ever-increasing interest, there are microenvironment-targeting strategies, which can be coupled with classical anti-leukemia therapies to improve the prognosis of relapsed/refractory patients, whose management represents an unmet medical need. In the Table 2, most important novel agents are summarized together with the corresponding targets.

Targeting drug	Name	Spectrum	Results	Status	Clinical Trials
Targeted cytotoxic drug					
CD33	gemtuzumab ozogamicin	CD33+	Relapse risk reduction (HR 0.81), 5 year-OS improvement (HR 0.90)	FDA approval	25 recruiting studies in association
	SGN-CD33A (vadastuximab talirine)	CD33+	Higher rate of death	*Terminated*	*NCT02785900*
	lintuzumab-Ac225	CD33+	Under investigations	Phase 1 & phase 1/2	NCT03441048, NCT03867682
CD123	IMGN632		Under investigations	Phase 1/2	NCT04086264, NCT03386513
Epigenic-directed therapies					
IDH1	ivosidenib	IDH1^{mut}	R/R AML: CR/CRi 30.4%, ORR 41.6% (phase 1)	FDA approval	9 recruiting studies alone or in association
IDH2	enasidenib	IDH2^{mut}	R/R AML: CR 19.6%, ORR 38.8% (phase 1/2)	FDA approval	12 recruiting studies alone or in association
HDAC	panobinostat		Limited benefit	Phase 3	NCT04326764
	vorinostat		Limited benefit	Phase 1 & phase 1/2	NCT03263936, NCT03843528, NCT03878524, NCT03842696
	belinostat		Under investigations	Phase 1	NCT03772925
	entinostat		Under investigations	Phase 2	NCT01305499
EZH2	DS-3201b		Under investigations	Phase 1	NCT03110354
DOT1L	pinometostat	MLL^{mut}	Under investigations	Phase 1/2	NCT03724084, NCT03701295
KDM1A	tranylcypromine		Under investigations	Phase 1	NCT02273102
	INCB059872		Under investigations	Phase 1	NCT02712905
BET	OTX015	MLL^{mut}	*Study withdrawn*	*Phase 1*	*NCT01713582, No further study*
	GSK525762		Under investigations	Phase 2	NCT01943851
BCOR	crizotinib	BCOR^{mut}	Under investigations	Phase 2	NCT02638428
BCL2	venetoclax	NPM1^{mut} IDH1/2^{mut}TP53^{mut}	In association to HMA in AML ineligible: CR/CRi 67%	Phase 3	56 recruiting studies in association
TP53	elesclomol	TP53^{mut}	Under investigations	Phase 1	NCT01280786
XPO-1	selinexor	NPM1^{mut}	Under investigations	Phase 1	NCT02091245, *NCT02093403, NCT02299518*
Kinase inhibitors					
FLT3	midostaurin	FLT3^{mut}	Front line FLT3^{mut}-AML: OS 74.7 months (HR 0.78)	FDA approval	13 recruiting studies alone or in association
	sorafenib	FLT3^{mut}	Largest prospective studies but significative toxicities	Phase 2	12 recruiting studies in association
	gilteritinib	FLT3^{mut}	Phase 3: OS 9.3 months vs 5.6 months (HR=0.64), CR/Cri 34%	FDA approval	9 recruiting studies in association
	quizartinib	FLT3^{mut}	Limited benefit	FDA refusal	9 recruiting studies in association
	crenolanib	FLT3^{mut}	Sensibility despite FLT3-D835 mutation	Phase 2 & 3	NCT03258931, NCT03250338, NCT02400255
MAPK	vemurafenib	NRAS^{mut}	Under investigations	Phase 1 & 2	NCT03878524, NCT02638428
	pazopanib	NRAS^{mut}	Under investigations	Phase 2	NCT02638428
	tivozanib	NRAS^{mut}	Sensibility	Pre-clinical	None
mTOR	everolimus	RUNX1^{mut}	Under investigations	Phase 1/2	NCT02109744
PIK3C-mTOR	dactolisib	RUNX1^{mut}	Sensibility	Pre-clinical	None
VEGFR	cediranib	MLL^{mut}	*No confirmed responses*	*Phase 2*	*NCT00475150, no further study*
BTK/ITEC	ibritinib	FLT3^{mut} ± NPM1^{mut}	Under investigations	Phase 2	NCT02351037, NCT03267186
SYK	entospletinib	FLT3^{mut}	Under investigations	Phase 1/2	NCT02343939, NCT03135028, NCT03013998
JAK2	ruxolitinib	JAK2^{mut}	Under investigations	Phase 1/2	NCT03558607, NCT02257138
	ruxolitinib + decitabine	BCOR^{mut}	Under investigations	Phase 2	NCT04282187
	ruxolitinib + venetoclax	RUNX1^{mut}	Under investigations	Phase 1	NCT03874052
IRAK1/4-FLT3-JAK2	pacritinib		*Anti-AML activity but stopped due to financial constraints*	*Terminated*	*NCT02532010*
IRAK4	CA-4948		Under investigations	Phase 1	NCT04278768, NCT03328078

Table 2. Novel agents recently approved for AML[159]

3 Crosstalk AML-Endosteal niche

Numerous data indicated that during AML, malignant cells similarly to normal HSCs, engage a bidirectional crosstalk with the BM niche. This specific interdependency hijacks the normal BM niche components generating a leukemic niche that is to the detriment of normal hematopoiesis favoring instead AML cells proliferation, survival and immune evasion[160].
Interestingly, several studies sustain that not only AML can alter the niche but there is also a prominent role of specific alterations of the niche which can act as predisposition events in the pathogenesis of myeloid malignancies [161]. In next paragraphs, a brief overview regarding these aspects will be presented. For more details we referred to our review reported in Chapters 2 currently under revision on Hemasphere.

3.1 Niche remodeling by AML cells

As explained in depth in the work of Tettamanti and Pievani recently published[162], the onset of AML causes profound changes in both structural organization and biochemistry of the BM niche, facilitating leukemia maintenance, spreading and escape. In this chapter, we will discuss major alterations identified in AML patients' BM and confirmed in AML murine models, such as enhanced micro-vessel density, neuropathy, altered cellular composition, and bone remodeling [161,163,164]. Moreover, we will describe BMSCs changes found in AML patients.
Intravital microscopy on an AML murine model showed a significant remodeling of vascular endothelium with a reduction of blood vessel number [44] and blood flow, which in turn generate a hypoxic milieu, especially in the endosteal region [165]. This hypoxic condition results in HSC loss and drugs and immune cell trafficking impairment[166,167] favoring AML engraftment and survival[168]. Proinflammatory factors, anti-angiogenic cytokines and nitric oxide (NO) released by AML cells have been identified as the main effectors of the vasculature tampering[169].

Another alteration detected in AML patients and subsequently confirmed in AML murine model is the disruption of nerve fibers around arterioles in BM. The reduction of sympathetic nerves favors the proliferation of NES^+ BMSCs while reducing NG2+ periarteriolar cells, promoting AML maintenance[44,170].

A further feature characterizing AML patient is the increased number of adipocytes detected in their BM, which is strictly related to poor prognosis. Murine models showed that AML cells alter BM niche composition increasing adipocyte number for their own nourishment, at the expense of normal HSC [171,172]. Indeed, AML cells modulate adipocyte metabolism, promoting lipolysis of triglyceride to fatty acid through induction of hormone-sensitive lipase. In these conditions, AML cells shift their metabolism toward fatty acid oxidation (FAO), obtaining the energy required for their proliferation. Moreover, fatty acid abundance increases PD-1 expression and reduce INF-γ secretion inhibiting effector T cells, while activating MAPK signaling to promote T-regs differentiation[173] and M2 macrophages polarization[174], creating an immunosuppressive microenvironment protective for leukemic cells.

The close correlation present between adipocyte and osteoblast number, due to their same origin from BMSCs, suggested that an increase in adipocytes could correspond to a reduction in osteoblast number. This hypothesis was confirmed by the defective bone turnover, with a diminished generation of bone tissue, detected in an AML murine model[175].

Furthermore, Kumar and colleagues showed AML cells reshape the BM niche into a leukemia-supportive mileu releasing exosomes which induce in BM stromal cells the expression of DKK1, a suppressor of normal hematopoiesis and osteogenesis as well as downregulate hematopoietic stem cell-sustaining factors. More precisely, this inhibitor blocks the osteo-lineage maturation, as highlighted by the reduced expression of genes involved in osteogenesis (OCN) favoring AML growth *in vitro* and *in vivo*[176]. The decreased plasma levels of osteocalcin (Ocn) in AML patients confirm the impaired osteogenesis found in AML mice models[176].

As BMSCs play a pivotal role in hematopoiesis regulation, several studies focused on the possible effects that AML blasts may have on these cells. There are several discrepancies among existing data on the features of BMSCs isolated from patients with AML. In early *in vitro* studies, BMSCs obtained from BM of AML patients (AML-BMSCs) exhibited altered morphology[177,178], lower clonogenic capability[177,179] decreased proliferation[177,179], reduced osteogenic potential[177,180,181], increased adipogenic differentiation[180], higher senescence[179] and impaired ability to sustain normal hematopoiesis[177–179]. Other studies showed instead that AML-BMSCs are normal in terms of morphological[180,182], proliferative[181], differentiative. Beyond these diverging results, Pievani and colleagues evaluated *in vivo* the possible corruption of BMSCs induced by AML cell exposure, demonstrating that they present a blockage in the first steps of osteogenic differentiation[183]. Their results agree with data obtained from Kumar and Frisch, reported above.

Moreover, transcriptional and epigenetic analyses have been performed on BMSCs to identify which mechanisms can be affected by leukemic cell presence. These analyses brought to light that AML cells reprogram BMSCs transcriptome, confirming the increased expression of previously mentioned genes such as CXCL12 and IL-6 and allowing the identification of additional factors that were altered by the presence of AML cells, such as IL-8, IL-1β, CCL2, prostaglandin E2 (PGE2), IDO-1, and pro-survival genes[168,177,181,184,185]. These results strengthen the importance of CXCL12/CXCR4 axis. CXCL12 and its receptor CXCR4 favor LSCs migration to the BM niche and support their survival, proliferation and drug resistance [186]. Instead the upregulation of IDO induces immunomodulation and immunosuppression, creating a protective microenvironment for AML[187]. More precisely, it increases the capacity to induce differentiation of T cells toward Tregs [188,189], promotes suppression of lymphocyte proliferation *in vitro* and diminishes secretion of pro-inflammatory cytokines such as IL-10[190].

An interesting mechanism used by corrupted BMSCs to protect AML cells from chemotherapy is the transfer of mitochondria to leukemic cells through endocytic pathways or tunneling

nanotubes (TNT). This passage allows AML cells to increase their ATP production and then reduce oxidative stress caused by drugs [191]. Moreover, *in vitro* co-culture studies demonstrated that BMSCs promote chemoresistance up-regulating Bcl-2 and Bcl-X and activating the c-Myc pathway in AML cells[192].
Another mechanism used by BMSCs to crosstalk with AML cells is the Notch signaling. Activation of Notch signaling induce AML cell proliferation, survival and chemoresistance in co-cultures with BMSCs[193]. Furthermore, BMSCs isolated from patients with AML possess a different Notch expression pattern with higher levels of Jagged1 and Notch1 and more pronounced chemoprotective features compared to normal[179,179,194]. Notch blockade by pharmacological inhibition reduces AML proliferation and abrogates BMSCs-mediated AML resistance to chemotherapeutic agents through the modulation of AKT, STAT3 and NFKB[193]. For this reason, the study of Notch signaling role in AML/BMSCs crosstalk is paramount.

3.2 Niche-driven initiation of myeloid malignancies

While early studies considered genetic lesions in the hematopoietic cells the only cause of leukemia onset, in the present day, after the elucidation of the primary role of the niche, several works focused on alterations that affect the non-hematopoietic compartment and their role in the malignancy initiation. Among them, pioneer studies were performed by Walkley and colleagues. Firstly, they generated a murine model in which retinoic acid receptor γ was knocked-out (RAR $\gamma^{-/-}$) in the hematopoietic and stromal compartments. This model showed a myeloproliferative (MDS) syndrome characterized by a significant increase in granulocyte/macrophage progenitors within mouse BM. This aberrant phenotype is observed even when healthy (WT) hematopoietic cells were transplanted into RAR $\gamma^{-/-}$ mice, but not in WT mice transplanted with RAR $\gamma^{-/-}$ hematopoietic cells[3]. Following, similar results were obtained by deleting in the stromal compartment and myeloid cells the gene

encoding the retinoblastoma-associated protein ($Rb^{-/-}$), a central regulator of the cell cycle[195]. Also in this case, the resulting MPN-like phenotype occurs selectively in $Rb^{-/-}$ mice transplanted with WT HSC, demonstrating that it was the consequence of Rb-dependent crosstalk between myeloid-derived cells and the altered microenvironment[195]. These works were only the beginning of a new generation of studies that, over time, have identified key components of the BM niche that, if mutated, can cause malignancy initiation. Indeed, in 2010 Raaijmakers' laboratory showed that the deletion of miRNA processing endonuclease *Dicer1* selectively in osteoprogenitors results in a MDS-like disease, that can sporadically evolve in AML[4] and cannot be transmitted through HSC transplant[4]. Interestingly, while deletion of *Dicer1* in mature OB does not induce disease onset, its deletion in BMSCs cause a reduced expression of *Sbds*, the gene mutated in Shwachman–Bodian–Diamond syndrome, a human BM failure associated with leukemia predisposition. Moreover, *Sbds* deletion in osteoprogenitors induces a genotoxic stress in HSPC through the increase of p53 signaling which in turn stimulates the secretion of inflammatory molecules S100A8/9, favoring leukemogenesis[4,196]. Throughout the years, other alterations of non-hematopoietic cells able to give rise to MDS have been identified, such as the conditional expression of mutant protein tyrosine phosphatase non-receptor type 11 (Ptpn11), which is a positive regulator of RAS signaling, in BMSCs and osteoprogenitors[197], or the constitutive activation of parathyroid hormone receptor (*PTHR*) in OB which induce MLL-AF9 driven AML[198].

All these reports indicate that microenvironment alterations can be the sole cause of myeloid neoplasm development in mice. However, a causative function played by BMSCs and their progeny in the pathogenesis of AML in humans still needs to be proven. Several works focused on the central role of osteoblasts in myeloid malignancy initiation and progression. Indeed, genetic depletion of osteoblasts in an AML murine model favor disease spreading, as highlighted by increased circulating blasts and shorter animal survival, maintenance of osteoblast levels restored normal marrow functions. Indeed, the inhibition of the

synthesis of duodenal serotonin, a hormone that suppresses osteoblast numbers, reduced tumor burden and prolonged mice survival[163]. These results confirm that osteoblasts play a fundamental role in propagating leukemia in the marrow and therefore may represent a therapeutic target to induce hostility of the niche to leukemia.

Among the studies which evaluated the effects of alterations in the non-hematopoietic compartment of the murine BM on leukemia development, many focused on Notch signaling.

One of the earliest works concerning the role of Notch pathway alterations in the non-hematopoietic BM cells on the myeloid neoplasm onset, showed that Adam10 deletion in the BM compartment, with the consequently reduction of NICD release in the cytoplasm, triggers granulopoiesis and expand HSC pool favoring a myeloproliferative phenotype [199]. Kim and colleagues demonstrated that an MPN-like phenotype occurs also in mice presenting Mind bomb 1 (Mib1) knockout in the non-hematopoietic compartment. The absence of this E3 ubiquitin ligase, which regulates endocytosis of Notch ligands, causes the accumulation of immature granulocytes, hepato-splenomegaly, anemia, and leukocyte infiltration. The transplantation of WT BM cells into Mib1-KO mice results in de novo MPN[72]. Further works revealed that loss of Notch signaling and, consequently, of its binding to the Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ) causes an augmented concentration of miRNA-155 in endothelial cells. The consequence of this increase is the hyper-activation of NF-kB signaling and the augmented release of proinflammatory cytokines (GM-CSF, TNF-α), due to the degradation of its inhibitor, kB-RAS1, after miR-155 targeting[200]. miR-155 was found to be up-regulated also in patient-derived AML blasts. Lastly, constitutive activation of β-catenin in osteoblasts leads to AML development in mice. In detail, an increased concentration of β-catenin stimulates FoxO1 signaling which consequently increases the expression of Jagged-1 on osteoblasts surface. The binding between Jagged1 and Notch receptors on HSC alters the differentiation potential of myeloid and lymphoid progenitors, leading to the development of AML[5,201]. All these

findings demonstrate that global (canonical and non-canonical) deregulation of Notch signaling in osteolineage cells can support the development of a myeloproliferative-like syndrome driven by both cell-autonomous and non-cell-autonomous mechanisms[200], and therefore suggest the potential therapeutic use of Notch in myeloid neoplasms.

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SCOPE OF THESIS

The purpose of this PhD project is to substantiate the presence of major alterations, particularly in the osteogenic differentiation capability, on BMSCs exposed to AML cells.
The first chapter provides a general introduction about the role and composition of the BM niche and its involvement in the development of myeloid malignancies. Specifically, this chapter elucidates fundamental interactions so far discovered between the BM stromal niche and the healthy or malignant hematopoietic compartment, focusing on the central role that BMSCs and their osteogenic progeny have on the regulation of hematopoiesis during homeostasis and leukemogenesis.
In the second chapter I reported a review (under revision on Hemasphere journal) where we discussed the contribution of BMSCs and their osteogenic progeny in supporting the initiation, progression, and chemoresistance of myeloid malignancies. We summarized the current knowledge about specific alterations of the stromal BM niche which can act as predisposition events, facilitating mutant myeloid cell survival and expansion as well as contributing to malignancy progression and providing protection from chemotherapy. We also presented the most recent advances in identifying BMSC subset heterogeneity and cellular hierarchy by single-cell technologies and their impact on mapping the remodeling of BM during AML.
In the third chapter I reported an article (published in Haematologica journal in 2021) in which we used *in vitro* assays and *in vivo* heterotopic osteogenesis transplantation systems to compare AML patient- and normal donor-derived BMSC features. Here, we were able to demonstrate that although AML-BMSCs did not show evident alterations compared to normal BMSCs in terms of morphology, immunophenotype, proliferative and differentiation potential *in vitro*, when their ability to differentiate into osteoblasts or to generate a complete stromal niche was evaluated with the two *in vivo* systems, AML-BMSCs highlighted significant alterations. Indeed, AML-BMSCs exhibited a reduced bone formation capacity and developed an

osteoprogenitor-rich niche with the presence of Osterix-expressing osteoprogenitor cells. Moreover, we could also show that AML-BMSC-derived ossicles contained a significantly increased fraction occupied by adipocytes. These results demonstrate that BMSCs, even when removed from their pathological environment, maintain an intrinsically abnormal differentiation pattern with altered osteogenesis and increased adipogenic potential.

In the fourth chapter I reported a paper (manuscript in preparation) in which we studied, using *in vitro* and *in vivo* systems, the effect of AML cells on the osteogenic commitment of normal BMSCs and the mechanism involved in this detrimental crosstalk. We found that BMSCs exposed to AML cells acquire defects in osteogenesis, with altered expression of osteogenic markers *in vitro* and reduced bone formation *in vivo*. The direct interaction between AML cells and BMSCs is indispensable in influencing BMSC osteogenic differentiation and AML-mediated Notch activation in BMSCs seems to contribute to the ineffective osteogenic commitment.

Finally, the fifth chapter reports general conclusions, summarizing the main findings and describing future perspectives.

CHAPTER 2: A QUESTION OF FRAME: THE ROLE OF THE NICHE IN MYELOID MALIGNANCIES

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AUTHOR CONTRIBUTIONS

C.T, B.R., and A.P. wrote the manuscript; C.T. prepared the figures; A.B. and M.S. revised and edited the manuscript. All authors have read and agreed to the final version of the manuscript.

ABSTRACT

Until a few years ago, the onset of acute myeloid leukemia (AML) was entirely ascribed to genetic lesions in hematopoietic stem cells. These mutations generate leukemic stem cells which are known to be the main ones responsible for chemoresistance and relapse. However, in the last years, increasing evidence demonstrated that dynamic interplay between leukemic cells and bone marrow (BM) niche is of paramount relevance in the pathogenesis of myeloid malignancies, including AML. Specifically, BM stromal niche components, such as mesenchymal stromal cells (MSCs) and their osteoblastic cell derivatives, play a key role not only in supporting normal hematopoiesis but also in the manifestation and progression of myeloid malignancies. Here we reviewed recent clinical and experimental findings about how genetic and functional alterations in MSCs and osteolineage progeny can contribute to leukemogenesis and how leukemic cells in turn generate a “corrupted” niche able to support myeloid neoplasms. Moreover, we discussed how the newest single-cell technologies may help dissect the interactions between BM stromal cells and malignant hematopoiesis. The deep comprehension of the tangled relationship between stroma and AML blasts and their modulation during disease progression may have a valuable impact on the development of new microenvironment-directed therapeutic strategies, potentially useful for a wide cohort of patients.

1. INTRODUCTION

Acute myeloid leukemia (AML) is a hematopoietic malignancy harbored from hematopoietic stem cells (HSCs) and progenitor cells. The annual incidence is around 2.4 cases per 100,000 individuals and it increases progressively with age, to a peak of 12.6 per 100,000 in adults of 65 years of age or older[1]. A proportion of AML cases does not present as a de novo disease but represents the clinical evolution of clonal hematopoietic stem cell disorders such as myelodysplastic syndromes (MDS) or myeloproliferative neoplasms (MPN). In recent years, there have been major advances in our understanding of AML, including new knowledge about the molecular pathogenesis, leading to an update of the disease classification[2,3], technological progress in genomic diagnostics and assessment of minimal residual disease[4,5,] and the successful development of new therapeutic agents[6]. Disease-specific chemotherapy regimens and consolidation with hematopoietic cell transplant (HCT) have enhanced AML outcomes, reaching an overall survival of approximately 60% at 5 years across different studies[7,8], and chemo-free regimens have opened new possibilities for elderly patients not eligible for chemotherapy treatment[9]. Despite that, AML patients still face a risk of chemoresistance and relapse that severely affects the outcome. Furthermore, the application of new targeted therapies into clinical practice remains challenging due to the high complexity of this disease, including multiple driving mutations and the coexistence of several competing tumorigenic clones. These persistent difficulties necessitate the identification of innovative therapeutic approaches that are effective for a larger cohort of AML patients.

The main cause of relapse in AML patients is the presence of chemoresistant leukemic stem cells (LSCs) nested in the bone marrow (BM) niche[10]. LSCs are characterized by quiescence and self-renewal capability, which make them able to induce AML onset when transplanted in secondary and preferentially tertiary recipient mice and to sustain cell survival in optimized ex vivo co-culture systems[11]. LSCs arise from the accumulation of somatic DNA mutations in HSCs in genes involved in quiescence, cell

cycle and cell proliferation (FLT3, RAS, P53, c-KIT e STAT3), self-renewal potential, differentiation capability (NPM1 and CEBPA), and epigenetic regulation such as DNA-methylation related genes (DNMT3A, TET2, and IDH-1/2)[12]. Advances in cancer genomics have revealed that most AML genomes display small numbers of mutations, which are acquired in a stepwise manner (named the two-hit theory)[13]. LSCs therapeutic targeting and elimination are mandatory to improve the dismal AML outcome. However, LSCs not only share surface markers with HSCs and AML blasts, but also present a large heterogeneity within and among patients, and thus the identification of therapeutic LSC-specific targets is challenging[14].

The BM niche is a complex network of molecular and cellular entities composed of mesenchymal stromal cells (MSCs), endothelial cells, osteoblastic cells, nerves from the sympathetic nervous system, and accessory cells (e.g., macrophages, megakaryocytes, T-cells). It plays a role not only in BM homeostasis but also in hematological malignancies, such as myeloid diseases[15]. Indeed, malignant cells reside within the niche and extensively communicate with its components. These interactions contribute to the initiation of malignancy, support of disease progression, resistance to chemotherapy, and loss of normal hematopoiesis.

This review describes the latest findings regarding the interplay between myeloid malignancies, such as AML, MDS and MPN, and the BM stromal niche, focusing on clinical and experimental aspects that support the pivotal role of the MSCs and their osteoblastic cell derivatives specifically in supporting disease initiation and progression. Unraveling the molecular basis of these interactions, by using new sophisticated imaging and sequencing technologies, is a prerequisite for identifying new targetable cell-extrinsic factors in the BM niche that, in addition to the classical targeting of leukemic cell-intrinsic mechanisms, can improve the therapeutic benefit for these patients.

2. ALTERATIONS OF BM NICHE IN MYELOID MALIGNANCIES: CLINICAL EVIDENCE

The BM niche can become altered during hematopoietic malignancies, as it has been demonstrated in multiple murine models of leukemia and has been very well-described in patients with multiple myeloma[16,17]. Regarding myeloid diseases, the most striking evidence of BM niche alteration in patients is the BM fibrosis that occurs during the development of MPN, especially in myelofibrosis (MF). Although intrinsic MSC alterations have been observed in MF patients[18,19], pathological HSCs play a pivotal role in subverting the BM niche[20]. This concept is reinforced by data from patients that recovered after HCT where a complete response is defined by the complete regression of BM fibrosis[21].

In AML patients, some morphological features of the BM have been described that correlate with the outcome. In the study of Lu and colleagues, the authors analyzed the size of marrow adipocytes in BM sections from 70 patients with primary AML, observing a correlation between increased amounts of small BM adipocytes and a poorer prognosis[22]. Another study showed that BM biopsies of AML patients presented a significant increase of CD271+ MSCs which in turn enhanced the production of reticular fibers[23], commonly associated with therapy failure and decreased overall survival[24]. Several studies reported an increased number of vessels and other vascular abnormalities in AML patient-derived BM biopsies, raising the interest in anti-angiogenic therapies also in AML[25]. However, whether increased angiogenesis constitutes a prognostic factor for AML treatment response remains unclear. Furthermore, a decrease of osteocalcin (OCN), a noncollagenous protein strictly correlated with bone turnover, was found in BM and peripheral blood (PB) serum of AML patients, especially those with adverse-risk disease[26]. Notably, a reduced OCN level correlates to inferior overall survival, poor response to chemotherapy, and lower relapse-free survival in AML patients. Moreover, chemotherapeutic treatments can cause a restoration of OCN

levels as a consequence of an increase in osteoblast (OB) number in the BM[23,27].

The BM microenvironment is implicated in drug resistance not only by changing the pharmacokinetics of drugs by the alteration of BM vascular permeability[28], but also by specific signaling pathways triggered by the altered BM components, especially MSCs. Indeed, it has been extensively described how MSCs endow AML cells with resistance to several therapeutic agents, including standard chemotherapy and new molecular targeted agents such as Fms-like tyrosine kinase (FLT3) inhibitors (midostaurin, sorafenib, and quizartinib). Co-cultures of AML cells harboring internal tandem duplication of FLT3 (FLT3-ITD) together with MSCs protect blasts from FLT3 inhibitors through the alteration of cytokines, chemokines, RAS/MAPK, and ERK signaling pathways[29]. However, the precise population of cells and molecular mechanisms involved in chemoresistance have only been partially elucidated.

An important demonstration of the role of the BM niche in protecting leukemic cells from chemotherapy comes from the clinical use of the CXCL12/CXCR4 axis inhibitors. Indeed, different groups have tried to boost chemotherapy activity by stripping the LSCs from their niche, upon administration of granulocyte colony-stimulating factor (G-CSF) or plerixafor in combination with antileukemic treatments, with promising results[30,31]. In particular, Uy and colleagues demonstrated an increased overall survival and relapse-free survival in AML patients treated with plerixafor in combination with chemotherapy31. A similar strategy was adopted in combination with hypomethylating agents such as decitabine or azacytidine[32] or in combination with targeted drugs like sorafenib[33]. Treatment with sorafenib, G-CSF, and plerixafor significantly increases the mobilization of blasts and CD34+/CD38- stem/progenitor cells, and the overall response rate as well.

3. THE ROLE OF MSCS AND THEIR OSTEOLINEAGE PROGENY IN NORMAL AND MALIGNANT HEMATOPOIESIS

In the last years, our understanding of HSCs and their niche has increased exponentially thanks to technological advancements, including the use of reporter mice, refinement of HSC markers, single-cell RNA sequencing (scRNA-seq), CyTOF, full bone sections as well as live animal imaging. Within the BM niche, HSCs are found predominantly located in perivascular regions, including sinusoidal and arteriolar vessels[34,35]. Only a minor fraction of HSCs resides near trabecular and cortical bone surfaces[36]. However, HSCs are motile within the niche and cannot be strictly localized within a specific area[37]. Multiple cell types, including stromal and endothelial cells, populate perivascular regions. The stromal fraction includes MSCs capable of differentiating to OBs, chondrocytes, and adipocytes. MSCs differ between the distinct BM regions, where periarteriolar MSCs display a propensity to undergo osteogenesis, whereas sinusoidal MSCs show an enhanced adipogenic profile[38–40].

BM MSCs co-localize closely with HSCs and regulate HSC homeostasis through the production of soluble factors, including CXCL12, angiopoietin (ANG), and stem cell factor (SCF). Multiple MSC subsets with distinct impacts on HSC behavior have been identified. CD271+/CD146-/low MSCs are bone-lining cells that support long-term, quiescent HSCs in areas with low oxygen tension. In contrast, CD271+/CD146+ MSCs are located in the perivascular region where they support more proliferative HSCs[41]. Furthermore, a specific type of perivascular MSCs, Nestin+/NG2+ MSCs, produces high levels of CXCL12 and ANG, and their depletion results in the loss of HSCs[42].

Osteolineage cells, including preosteoblasts and osteoblasts, were among the first component of the niche to be associated with hematopoiesis. Expansion of osteolineage cells by targeted activation of parathyroid hormone (PTH) receptor 1 or by conditional inactivation of bone morphogenic protein (BMP) receptor type 1A is associated with an increase of HSCs through

Notch1/Jagged1 or N-cadherin-mediated cell adhesion, respectively[43,44]. Conversely, conditional ablation of osteolineage cells correlates with a loss of HSCs in the BM[45]. OBs could be the preferred niche for specific subtypes of HSCs that have distinct functional properties. Specifically, dormant HSCs resistant to myeloablation stress are located near the endosteal region, in contact with N-cadherin-expressing stromal cells with osteogenic potential[46].

The precise mechanisms used by OBs to regulate HSC maintenance and functions are still unclear. Among these strategies, there is the maintenance of HSC quiescence through Notch1/Jagged1[43], ANG-1/tyrosine endothelial cell kinase 2 (Tie2) signaling[47], thrombopoietin(TPO)/MPL axis[48], and non-canonical Wnt signaling regulated by Frizzled 8[49]. Moreover, OBs secrete several molecules involved in HSC regulation, such as G-CSF, type I collagen (COL-I), OCN, and osteopontin (OPN)[50,51]. In addition, osteolineage cells are fundamental to the maintenance of the homeostatic Ca^{2+} concentration, which is pivotal to the enrollment of circulating HSCs from blood to the BM niche[52]. Using CyTOF on BM stromal cells, Severe et al recently demonstrated that a subset of osteolineage cells expresses key hematopoiesis regulatory cytokines including SCF and stromal cell-derived factor 1 (SDF1)[53]. However, conditional deletion of Scf and Cxcl12 from OBs did not affect hematopoiesis, leading to reconsidering their role in the maintenance of HSCs[54,55].

A recent scRNA-seq study of BM stromal cells identified two distinct osteolineage cell subsets: OLC-1 and OLC-2. OLC-1 cells, which include early osteoprogenitors, express higher levels of key HSC regulatory genes[38]. The regulation of hematopoiesis by osteolineage cells depends indeed on their differentiation state. Mature OBs may be less important for HSC maintenance but are involved in the regulation of committed hematopoietic progenitor cells. In particular, the selective elimination of osteoblasts negatively affects the pool of early lymphoid precursors, without effects on HSCs[56].

Osteolineage cells have emerged also as critical regulators of the development and evolution of myeloid malignancies, including AML[57]. Imaging studies in mice have shown that leukemia cells

primarily home to and engraft close to the endosteal region where are protected from chemotherapy-induced apoptosis[10,58]. Proof-of-principle experiments using transgenic mice carrying genetic alterations in BM stromal cells and their osteoblastic cell derivatives suggested their contribution to the initiation and progression of myeloid malignancies.

4. BM MICROENVIRONMENT ALTERATIONS OR HSC MUTATIONS: A CHICKEN AND EGG SITUATION

AML is characterized not only by the blocking of HSC differentiation and the accumulation of blasts but also by alterations in the non-hematopoietic compartment of the BM microenvironment, which sustain leukemogenesis while suppressing normal hematopoiesis. MSCs and their progeny can contribute to leukemogenesis at least in two different ways: they can acquire mutations or functional alterations, caused by aging and chronic inflammation, that predispose for malignancy development, or they can be “corrupted” by transformed hematopoietic cells, facilitating disease manifestation and/or progression. Both these contributions can coexist during leukemia initiation and expansion.

4.1 The transformed niche as a predisposing factor for myeloid diseases: evidence from genetic animal models

Pioneering studies on reciprocal BM transplantation using mouse models carrying mutations in non-hematopoietic or hematopoietic cells demonstrated the potential of dysregulated stroma to drive the initiation of myeloid diseases, including MPN, MDS, and AML. For instance, mice with retinoic acid receptor-γ (RAR-γ) deficient microenvironment transplanted with wild-type (WT) BM rapidly developed MPN[59]. Similarly, the inactivation of the Retinoblastoma (Rb) gene, a central regulator of the cell

cycle, in both HSCs and BM microenvironment established a myeloproliferative disorder[60]. MPN was also developed after transplanting normal HSCs into mice carrying inactivation of Mind bomb 1 (Mib1), the primary regulator of endocytosis of Notch ligands, in BM stromal cells[61].

The first evidence that alterations of a specific BM stromal population represented by osteolineage cells can drive myeloid malignancies arose from the deletion of Dicer1, an RNAse III endonuclease, in osterix-expressing osteoprogenitors, which caused MDS-like disease with increased propensity to develop AML[62]. Dicer1 depletion in mature osteocalcin-expressing OBs did not result in a hematopoietic phenotype.

Mice presenting activating mutations in the Ptpn11 gene, encoding for the protein tyrosine phosphatase SHP2, in MSCs and osteoprogenitors but not in mature osteolineage cells showed the MPN development. These mice are characterized by the generation of a pro-inflammatory environment. Specifically, overproduction of the chemokine CCL3 by Ptpn11-mutated stromal cells can cause the recruitment of monocytes which secreted inflammatory cytokines such as IL-1β and hyperactivated HSCs, driving the disease progression[63].

Furthermore, constitutive activation of β-catenin in OBs leads to AML development in mice[64]. The increased nuclear β-catenin interacts with Forkhead box protein O1 (FoxO1), inducing the activation of Notch signaling and consequently increasing the expression of the ligand Jagged1 on OBs[65]. The subsequent aberrant activation of Notch signaling in HSCs induces their leukemic transformation and ultimately leads to AML development. Finally, it has been demonstrated that HSCs from β-catenin mutated mice acquired intrinsic alterations and, once they are transplanted in WT mice, they provoke AML onset[64].

More recent studies provided evidence that non-genetic changes, such as BM niche remodeling caused by premature or physiologic aging, can also foster myeloid cell expansion[66,67]. Genetic depletion of healthy mature OBs in an AML murine model causes an increase in circulating blasts and in tumor engraftment in the BM and spleen, an increase of myelopoiesis associated with a compromised B lymphopoiesis and

erythropoiesis, and shortened survival[27]. On the other hand, maintenance of the OB pool treating mice with an inhibitor of the duodenal serotonin synthesis increased mice survival, reducing circulating blasts and restoring the normal marrow function[27]. Similar results were obtained by Bowers and colleagues which demonstrated that OB ablation significantly accelerates leukemia development and reduces the survival of transgenic BCR-ABL mice[68]. Interestingly, the role of OBs in leukemogenesis seems to be disease-specific. Indeed, constitutive PTH receptor activation in OBs increased bone remodeling, causing TGF-β release from bone, which attenuated BCR-ABL CML progression but stimulated AML induced by MLL-AF9 oncogene[69]. In addition, OBs expand during the chronic phase of CML[70] and in contrast they are markedly reduced during the blast crisis[71], further suggesting that they are differentially affected in AML and CML.

Osteolineage cells are not the only BM cell type involved in leukemogenesis. Loss of canonical Notch signaling in endothelial cells leads to MPN-like disease, through constitutive activation of mir-155 and NFKB signaling[72]. Deletion of signal-induced proliferation-associated gene 1 (Sipa1) in mesenchymal and endothelial cells also results in MPN[73].

Despite all these studies demonstrating that BM populations, particularly osteolineage cells, can lead to a preleukemic myeloid disease when mutated or dysfunctional in mice [FIG.1], similar evidence is still missing in patients.

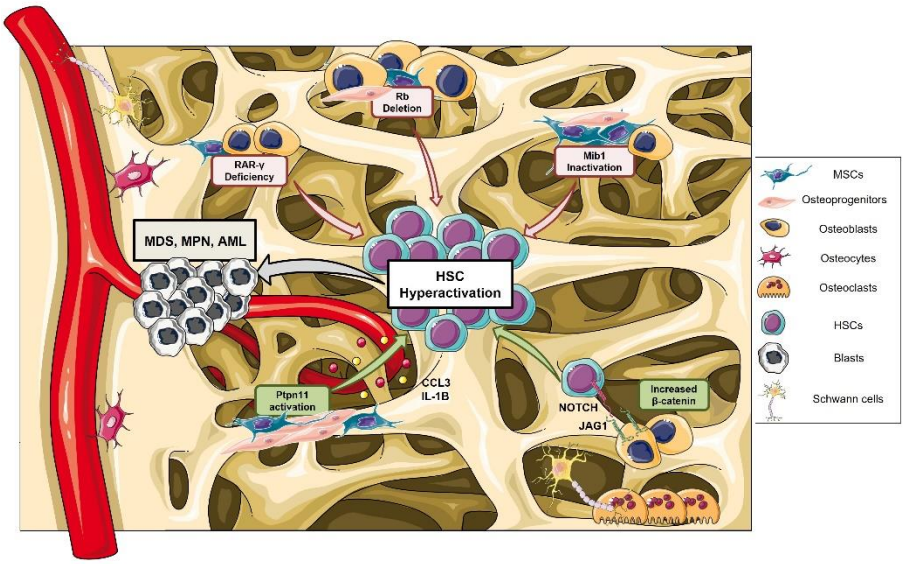

Figure 1. BM stromal cell alterations that induce myeloid malignancies.
Schematic representation of altered mechanisms or mutated genes in BM stromal niche components linked to the initiation of myeloid malignancies, such as myelodysplasia (MDS), myeloproliferative neoplasm (MPN), or acute myeloid leukemia (AML) in mice. Deletion of Retinoblastoma (Rb), deficiency of retinoic acid receptor-γ (RAR-γ), and inactivation of Mind bomb 1 (Mib1) genes in the stromal compartment led to MPN development. Similarly, activating mutation in tyrosine phosphatase SHP2 protein (encoded by Ptpn11) in osteoprogenitors increases the release of chemokine ligand 3 (CCL3) and interleukin (IL)-1β hyperactivating HSCs and gives rise to MPN. Moreover, activation of β-catenin in OBs causes Jagged1 upregulation on their surface, inducing Notch1 hyperactivation in HSCs and AML onset. A mutation of Dicer1 in osteoprogenitors leads to MDS. The Figure was partially generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

4.2 AML remodels the BM microenvironment favoring the generation of a self-reinforcing leukemic niche

4.2.1 Alterations in stromal cells isolated from AML patients

A possibility to approach the modifications of the human BM microenvironment in AML is to isolate and study the MSC populations derived from AML patients (AML-MSCs). Leukemic cells reprogram MSCs through cell-to-cell contact, secreted factors, and exosomes[74–76]. Although the phenotypic profile of cell surface markers defining stromal MSCs in the BM niche does not change between healthy subjects and myeloid malignancy patients, specific subpopulation proportions, functions, or molecular profiles appear to be altered. Cytogenetic abnormalities, such as hypodiploidy, chromosomal translocations, duplications, and deletions, have been observed in AML-MSCs. Interestingly, these mutations are different from those detected in the leukemic blasts of the same individual[77]. These alterations have also been confirmed by Kim and colleagues who described the presence of genomic instability and a global hypomethylation in AML-MSCs compared to the healthy ones[78]. The functional consequences of these MSC alterations are still debated. Several studies carried out on MSCs from patients with AML have noted normal proliferative capacity, colony-forming unit-fibroblast (CFU-F) capability, survival, and ability to support ex vivo hematopoiesis. In contrast, other studies showed defective hematopoietic supportive capacity, reduced expression of adhesion molecules (VCAM-1 and CD49F/ITGA6), increased apoptosis and senescence, and augmented production of inflammatory cytokines in MSC derived from patients with myeloid neoplasm[26,74,76,78,79]. In stromal cells from MDS and AML patients, the expression of molecules involved in interaction with HSCs is decreased whereas the population of CD271+ MSCs, which favors blast expansion through up-regulation of CXCL12, is increased26. MSCs sustain the survival and chemoresistance of AML blasts in several ways, such as

supplying pro-survival factors, providing metabolic substrates alternative to glucose, and rewiring their metabolism[80–82].

Recent bioengineering advances were made in recreating BM stroma through organ-on-a-chip devices that would allow the investigation of MSC-mediated chemoresistance mechanisms. Biomimetic scaffolds capable of mimicking bone extracellular matrix were used to study the effects of MDS/AML blasts on MSCs, reprograming their transcriptome toward novel pro-oncogenic activities[74,76].

The capacity of MSCs derived from patients with AML to differentiate into osteolineage cells is controversial. Several studies demonstrated that AML-MSCs present an impairment in the differentiation capability into the three mesodermal lineages, and in particular in the osteogenic differentiation[26,79]. Battula and colleagues showed that AML-MSCs present increased expression of tissue non-specific alkaline phosphatase (TNAP) and a switch from adipogenic to osteogenic differentiation, driven by a BMP-dependent mechanism. Similarly, AML cells upregulated the expression of connective tissue growth factor (CTGF) in normal MSCs and activated Smad1/5 signaling, inducing MSCs to differentiate into committed osteoprogenitors, but not mature OBs[83]. Our group recently demonstrated that AML-MSCs, even when removed from their pathological environment, retain an intrinsically abnormal differentiation pattern with altered osteogenesis. Using an *in vivo* system specific to assess their osteogenic potential, AML-MSCs exhibit a reduced capacity to form mature bone and develop an osteoprogenitor-rich niche with the presence of Osterix+/Osteocalcin- preosteoblasts and Osteocalcin+/Dentin matrix acid phosphoprotein 1- (DMP1-) immature osteocytes[84].

These data indicate that AML cells educated MSCs to overproduce functionally altered osteoprogenitors, although the reasons for this differentiation impairment remain to be elucidated.

4.2.2 Evidence of AML-induced stromal niche reprogramming from animal models

The BM niche becomes dramatically altered during AML progression, as clearly demonstrated in many murine models of leukemia. The endosteal vascular niche is severely remodeled through the secretion by blasts of anti-angiogenic and inflammatory cytokines, such as tumor necrosis factor (TNF) and chemokine ligand 2 (CXCL2), which impair stromal cells, endothelial cells, and OBs, reducing their sustain to normal HSC maintenance[85]. The ability of AML cells to alter BM vasculature is further supported by the extensive vascular remodeling observed in PDX models and BM biopsies from patients[25,28]. Interestingly, the rescue of endosteal vessels preserves HSCs and improves chemotherapy efficacy[85]. In addition, Xiao et al demonstrated that AML MLL-AF9 cell infiltration in the BM cause an expansion of Early B-cell factor 2+ (Ebf2+) MSCs with reduced Cxcl12 expression, which favor AML establishment[86].
Furthermore, leukemic myeloid cells alter the fine control of the osteogenic niche[70,71], increasing the number of functionally altered osteoprogenitor cells and immature OBs [FIG.2]. Blocking the terminal differentiation of MSCs into mature OBs seems to contribute to AML progression. In an MLL-AF9 AML model, the leukemia development causes local neuropathy, reducing the number of NG2+ periarteriolar cells, which are involved in the maintenance of HSC quiescence, and leading to the osteogenic differentiation of Nestin+ MSCs. Despite this commitment to osteoblastic lineage, these mice show a lack of terminally differentiated OBs and deficient bone mineralization[87]. Similar results were reported also for a JAK2V617F-MPN mouse model, in which sympathetic neuropathy causes a reduction of Nestin+ MSCs, which promotes the expansion of mutant HSCs and facilitates disease progression by loss of HSC-retention factor expression, including CXCL12, SCF, ANG1, and VCAM1[88]. Frisch and colleagues, using an immunocompetent murine model of leukemia, demonstrated that AML blasts alter directly normal OB functions. Engrafted animals presented decreased osteopontin-positive osteoblasts, loss of trabecular

structures, and lower serum levels of the bone formation marker OCN[71]. Osteoblastic inhibition by leukemia seems to be mediated by chemokine CCL3 which is increased in malignant BM cells, both in leukemic mice and patients[71]. Furthermore, FLT3-ITD-positive mice showed alterations in bone morphology suggesting a possible impact of aberrant oncogenic FLT3 signaling on the process controlling bone homeostasis[89,90].

Kumar and colleagues noted that AML patients present an increase in exosome secretion, which correlated with the reduction of OCN plasma levels. Moreover, transplantation of AML-derived exosomes induces in the murine BM microenvironment alterations similar to those described for mice engrafted with AML cells (e.g., block of osteolineage maturation and reduced bone formation)[75]. Interestingly, AML-derived exosomes transfer to the BM stroma the negative regulator of osteogenesis Dickkopf-1 (DKK1), which causes suppression of OB differentiation and supports a more rapid leukemia progression. Treatment of transplanted mice with DKK1 inhibitors significantly delays AML progression and increases their overall survival. In addition, AML-derived exosomes induce downregulation of HSC-supporting factors (e.g. CXCL12, KITL, IGF1) in BM stromal cells, reducing their capacity to support normal hematopoiesis[75]. Doron and colleagues demonstrated that AML cells can use extracellular vesicles to transmit endoplasmic reticulum (ER) stress and to promote the unfolded protein response in stromal cells, inducing their osteogenic commitment partially through the transfer of BMP2[91].

Recently, Galan-Diez and colleagues identified kynurenine (Kyn), a tryptophan catabolite, as a new promoter of the BM niche remodeling in AML. AML-secreted Kyn binds to the serotonin receptor 1B (HTR1B) on OBs, inducing a proinflammatory state characterized by increased expression of acute-phase protein serum amyloid A (SAA). SAA acts with a mechanism of positive feedback on leukemia cells by increasing the expression of indoleamine 2,3-dioxygenase-1 (IDO1), the rate-limiting enzyme for Kyn synthesis, thereby enabling AML progression. Genetic and pharmacological inhibition of kyneurin-

HTR1B interaction between leukemia cells and OBs hampers AML proliferation[92].

As the study of the BM niche contribution to leukemia development is hindered in humans, reliable models of the human leukemic niche are fundamental for the confirmation of these findings and their translational application. In this regard, humanized BM niche models offer a valuable alternative. Over the years, several models using different scaffold materials and implant techniques have been proposed to recreate a humanized BM microenvironment that allows studying the ability of human stromal cells to sustain the normal and/or leukemic engraftment and to evaluate the interactions between hematopoietic and non-hematopoietic compartments[74,76,93,94]. Battula and colleagues developed a human AML niche model, which consists of subcutaneous implantation of human bone fragments freshly collected from healthy subjects into the flanks of NSG mice transplanted with human AML cell lines. The authors demonstrated that mice transplanted with human AML cell lines presented a strong up-regulation of early-stage OB markers Runx2 and Osterix in both the endosteal surface and medullary cavity of implants, compared to those harvested from control mice, confirming an increased but incomplete osteogenic activity also in humanized leukemic BM[83].

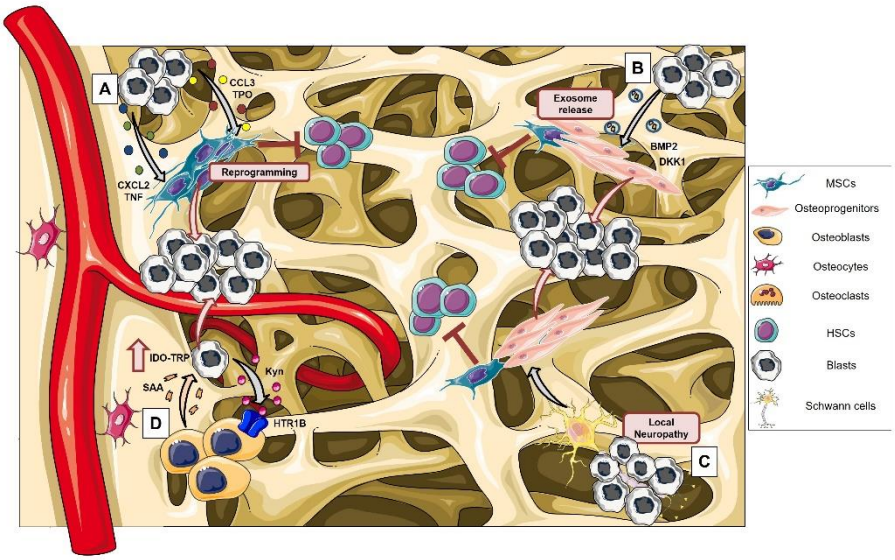

Figure 2. AML-induced alterations of the BM stromal niche.
Schematic representation of mechanisms by which AML cells reprogram MSCs and their osteoblastic cell derivatives, promoting the formation of a pro-leukemic niche. (A) AML cells secrete soluble factors, such as tumor necrosis factor (TNF), chemokine ligand 2 (CXCL2), Thrombopoietin (TPO), and chemokine ligand 3 (CCL3), which alter osteoblast formation from MSCs, impairing their hematopoietic stem cell (HSC)-supporting functions and favoring the proliferation of dysplastic cells. (B) Blasts release exosomes that are uptaken by mesenchymal stromal progenitors blocking their osteolineage development, reducing the support of normal hematopoiesis, and accelerating AML progression. (C) AML cells induce sympathetic neuropathy responsible for the reduction of HSC-maintaining NG2+ periarteriolar niche cells and the expansion of preosteoblasts which contribute to disease progression. (D) Leukemic cells secrete oncometabolites as kynurenine (Kyn) able to activate a self-reinforcing axis between AML cells and osteoblasts. Kyn, by binding to the serotonin receptor 1B (HTR1B), induces a proinflammatory state in osteoblasts that, through the acute-phase protein serum amyloid A (SAA), acts in a positive feedback loop on leukemia cells by increasing the expression of indoleamine 2,3-dioxygenase (IDO1), enabling AML progression. The Figure was partially generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

5. UNRAVELLING THE COMPLEXITY OF THE LEUKEMIC NICHE BY OMICS

Comprehensive mapping of the interactions occurring within the BM niche during AML pathogenesis and treatment would have a paramount impact on developing new therapeutical approaches targeting the leukemic microenvironment, aimed at improving the patient outcome. While the impact of AML on transcriptomic changes in the immune microenvironment has recently been addressed in patients[95], very little is known about the non-hematopoietic niche in human BM at the molecular level.

scRNA-seq[38,39,96] and spatially resolved transcriptomics[40] have recently consented to untwist the complexity of heterogeneous stromal progenitors in murine BM. Under homeostatic conditions, all these studies found considerable overlap between the identified niche populations. To examine how non-hematopoietic BM cells are altered during AML development, Baryawno and colleagues compared scRNA-seq profiles from mice with normal or MLL-AF9-knocked-in BM transplanted 6-10 months before analysis38. They identified 17 distinct BM populations at steady state, including 3 endothelial (sinusoids, arteries, and arterioles), 1 pericyte, 2 osteolineage (OLC-1 and OLC-2), 5 fibroblastic, 1 mesenchymal (LepR-MSCs), and 4 chondrocyte clusters. The MLL-AF9 mouse model showed significant alterations in the cellular composition of the BM microenvironment, with a marked decrease in osteolineage-differentiated LepR-MSCs and a concurrent increase in preosteoblasts. This suggests an impairment in osteogenic differentiation due to the induction of genes inhibiting the bone formation and calcification and is consistent with the decrease of bone formation and OB number reported in murine models of AML[75,87]. Even the adipogenic differentiation of LepR-MSCs was impaired as revealed by the downregulation of several adipocytic-related genes. This is in line with a previous study reporting defective adipogenesis in AML PDX models[97]. In addition, the presence of leukemic cells reduced the expression of regulatory molecules necessary for normal hematopoiesis in specific stromal subsets, including endothelial cells.

With a similar approach, Passaro and colleagues examined the changes affecting BM stromal cells during AML development using an AML PDX model[96]. They showed that major niche components, including CD31+ cells, Col1a1+ cells, and Nestin+ cells, which overlapped with the signature of endothelial, skeletal, and smooth muscle cells respectively, underwent gene expression changes upon AML engraftment. Integrating BM transcriptome and secretome, they described a deregulated proteomic signature similar to the one reported for AML patients98. Interestingly, several signaling mediators of the complement and coagulation cascade result downregulated, in agreement with clinical evidence of bleeding disorders and pro-thrombotic state in AML patients. Moreover, mediators associated with neutrophil degranulation are reduced, while chemokines involved in monocyte chemotaxis results increased, suggesting alterations in the regulation of innate immunity[96].

Similarly, a scRNA-seq study on BM stroma of mice with MPN reported a functional reprogramming of two specific MSC subsets into collagen-producing myofibroblasts, responsible for extracellular matrix deposition and fibrosis. Inflammation plays a critical role, as increased expression of alarmin heterodimer S100A8/S100A9 is related to stroma reprogramming from hematopoiesis support toward fibrotic transformation[99].

Very recently, scRNA-seq approaches have been applied to deconvolute the heterogeneity of MSCs in human BM, leading to similar findings compared to the murine experiments, revealing adipo-, osteo-, and chondrogenic clusters[100,101]. This represents the first step to unraveling alterations of BM stroma components during the development of myeloid malignancies in humans.

Whereas RNA-seq studies are useful, proteome-based studies would be essential to investigate molecules that are regulated by post-transcriptional mechanisms. Çelik and colleagues performed proteomic profiling of the noncellular soluble compartment of the BM microenvironment in patients with AML using the SOMAscan assay[98]. They found 91 upregulated and 77 downregulated proteins in the leukemic BM. The proteomic signature of the AML BM niche revealed perturbation of signaling networks, particularly those associated with chemokine and

cytokine signaling. Specifically, levels of proteins involved in bone homeostasis such as BMP10, CCL3, CX3CL1, osteopontin, endoglin, parathyroid hormone-like hormone, the secreted regulators of BMP signaling pathway, chordin-like protein 1 and matrix metalloproteinase 7 (MMP-7) displayed altered values.

Although more effort is necessary to unravel the BM stromal cell changes in different AML stages, all transcriptomic and proteomic data indicate a shift toward an accumulation of preosteoblasts that might be instrumental in disease evolution and should be further explored to identify novel therapeutic targets.

6. CONCLUSION AND FUTURE PERSPECTIVES

The studies reviewed herein, largely performed in animal models, highlight that leukemic myeloid cells and the BM stroma interact extensively, having mutual effects on each other. However, despite a growing set of evidence in patients sustains that leukemic cells can alter the stromal compartment by producing factors that corrupt the BM niche to their own advantage, the role of somatic mutations in BM stromal cells in leukemia initiation has not yet been confirmed in patients.

The elucidation of mechanisms behind the interaction between AML cells and BM stroma can represent a turning point in AML treatment. Indeed, despite the improvements in the management of this disease, AML patients still face a risk of chemoresistance and relapse that severely affect the outcome3. The ultimate goal of research into the hematologic malignancy niches is the development of therapies targeting the deregulated microenvironment, which, alone or in combination with the existing cell-intrinsic therapies, may increase leukemia susceptibility to treatment, leading to the complete eradication of all malignant clones. This could be accomplished by targeting the interactions between blasts and their niche, or blast-intrinsic pathways that influence the niche or directly the niche components. Since several mechanisms involved in leukemia

progression often overlap across multiple leukemic subtypes, niche-oriented therapeutic targets would be effective for a wide cohort of patients. Recent evidence about the main role of preosteoblasts on AML progression led to considering the crosstalk taking place between blasts and osteolineage cells as a potential target for therapy.

Our knowledge about cellular populations and molecular mechanisms of the BM niche involved in interactions with healthy and malignant hematopoiesis is still in its infancy. For this reason, the application of new imaging and sequencing technologies for the analysis of patients' BM samples represents a valuable option not only to shed light on the pathogenesis of myeloid neoplasms but also to identify possible novel therapeutic targets. Furthermore, monitoring the specific signature of the leukemic niche at diagnosis and during therapy could be exploited as a potential tool to predict response in clinical trial settings.

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CHAPTER 3: ACUTE MYELOID LEUKEMIA SHAPES THE BONE MARROW STROMAL NICHE *IN VIVO*

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Emerging evidence suggests that acute myeloid leukemia (AML) remodels the bone marrow (BM) niche into a leukemia-permissive microenvironment, while suppressing normal hematopoiesis.[1] The influence of AML on bone tissue architecture and osteogenic cell differentiation has been documented in murine models, however, the impact of patient-derived AML cells on human BM stromal cells (BMSC) has only been investigated using conventional *in vitro* approaches. We assessed the differentiation potential of AML-derived BMSC using two *in vivo* models that recapitulate the complex organization of the human hematopoietic niche. We found that BMSC derived from pediatric AML patients: i) exhibit a reduced mature bone formation, ii) develop an osteoprogenitor- rich niche, iii) generate bone/BM organoids with a higher adipocytic differentiation, and iiii) support the formation of osteoclasts in a similar proportion to normal donor controls. All these aspects may contribute to the inhibition of normal hematopoietic stem and progenitor cell development and propagate selective blast cell survival and expansion. AML is a heterogeneous disorder characterized by the clonal proliferation of blasts in the BM. Leukemic cells compete with normal hematopoietic stem cells for niche occupation and this results in alterations of the BM microenvironment and the generation of a “leukemic niche” that selectively supports the malignant clone.[1] AML-induced changes in the BM microenvironment have been confirmed in multiple *in vitro* and *in vivo* studies. Murine AML models have shown several alterations in the BM niche components (e.g., osteo-progenitors and osteoblasts) positively correlated with leukemogenesis.[2,3] Similarly, a decrease in osteoblast number has been observed in the BM of AML patients together with reduced osteocalcin serum levels.[4] Moreover, studies have also reported that BMSC, one of the main cellular components of the hematopoietic niche, derived from AML patients exhibit a number of molecular and functional alterations, such as translocations, gene expression modifications, reduced clonogenic potential, decreased proliferation, higher senescence, impaired *in vitro* adipogenic and osteogenic differentiation, increased support of leukemia growth, and imbalanced regulation of endogenous

hematopoiesis.[5-7] By contrast, other studies have reported that AML-BMSC display normal morphology and differentiation properties.[8] The humanized models are currently the only experimental system to reproduce an *in vivo* three-dimensional structure of the human BM niche, despite the limitations related to the potential interference of other components of recipient origins.[9] Several *in vivo* models have been described using normal or genetically modified human BMSC to generate a humanized BM niche that enables robust AML cell engraftment and allows evaluation of factors critical for the development/progression of leukemic cells within the niche.[9,10] The aim of our work was to evaluate if AML-BMSC have undergone significant changes in their capability to form bone and a BM niche after exposure to patient leukemia in the BM. We isolated and expanded *in vitro* BMSC from the BM of newly diagnosed pediatric AML patients (AML-BMSC) and healthy donors (HD-BMSC). Patients and healthy controls were age-matched. BMSC donor characteristics are summarized in the *Online Supplementary Table S1*. BMSC from both sources displayed a spindle-shaped, elongated morphology and formed discrete fibroblast colony-forming units with no differences in colony-forming efficiency (CFE) and in the number of cumulative population doublings (CPD) (Figure 1A-C). Similarly, BMSC derived from both sources revealed an identical *in vitro* immunophenotype consistent with standard criteria (Figure 1D). As a few studies have described impaired hematopoietic support capacities of AML-derived BMSC,[6] we investigated several cell-bound as well as secreted factors governing the hematopoiesis within the niche. We found significantly diminished mRNA levels of Kit-ligand (KITLG), while other hematopoiesis regulatory molecules such as VCAM1, Angiopoietin-1, CXCL12, and Jagged1 were unaffected (Figure 1E). We then evaluated the *in vitro* skeletogenic potential of AML-BMSC versus HD-BMSC by performing quantitative gene expression of known osteogenic and chondrogenic genes at baseline in monolayer cultures without the addition of any inducing factors. We found a similar expression in both AML-BMSC and HD-BMSC, except for SP7/Osterix levels which were significantly reduced in AML-

BMSC (Figure 1F). *In vitro* adipogenic, osteogenic, and chondrogenic differentiation assays showed normal tri-lineage differentiation potential for AML-BMSC population as proven by morphology, cytochemical staining, and upregulation of mRNA levels of tissue-specific markers (Figure 1G). The previously published data on *in vitro* AML-BMSC functional properties, mainly conducted using adult patient cohort samples, are contradictory. One of the reasons for such heterogeneity could be related to the age of patients. AML in young and adult patients should be considered differently since the biological and molecular characteristics of leukemic cells are different. In previous studies that included also pediatric cases, the reduction of adipogenic and osteogenic differentiation potential was correlated with AML characteristics at diagnosis and not to the patients' age.[8,11] In contrast, as our results confirm, the differential proliferative capacity of AML-BMSC is related to the age of the patients, with older patient samples displaying a reduction in proliferative ability when compared to younger patient samples.

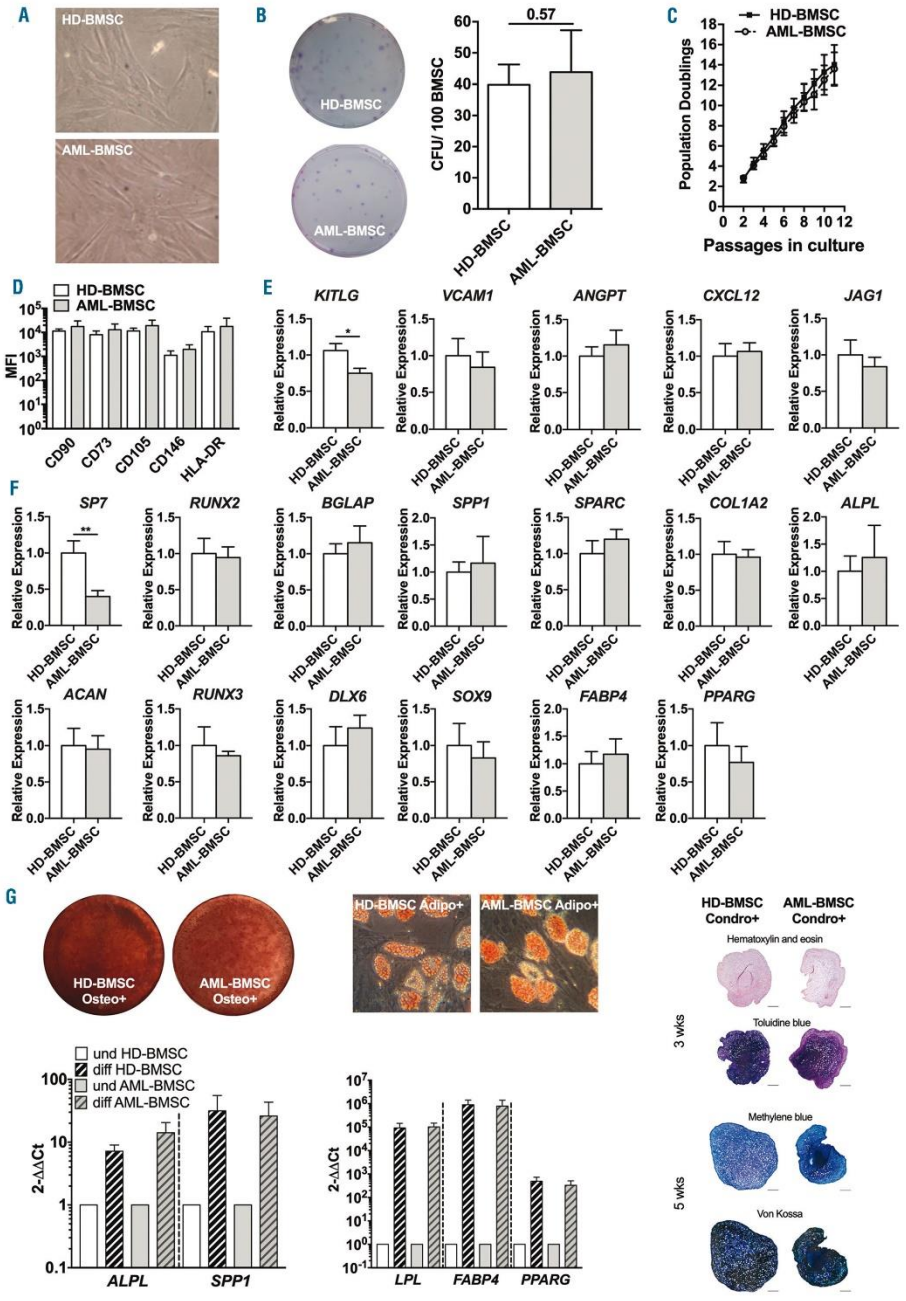

Figure 1. *In vitro* characterization of bone marrow stromal cells derived from bone marrow of pediatric acute myeloid leukemia patients and healthy donors. (A) Phase-contrast images depicting spindle-shaped morphology of bone marrow stromal cells (BMSC) derived from pediatric acute myeloid leukemia (AML) patients and healthy donors (HD). Magnification 40x. (B) Colony

forming unit (CFU-F) potential of AML- and HD-BMSC. Representative images of AML and HD CFU-F are shown in the left panel. Bar charts show mean ± standard deviation (SD) of CFU-F numbers normalized to 100 plated BMSC derived from AML (gray bar) *vs*. HD (white bar). (C) Proliferation measured as cumulative population doubling at each passage for AML-BMSC and HD-BMSC cultures. Bars show mean ± SD. (D) Expression of surface markers CD90, CD73, CD105, CD146, and HLA-DR on AML-BMSC and HD-BMSC at passage three. Bars represent mean ± SD of mean fluorescence intensity (MFI) values. (E) Quantitative RT-PCR-based analysis of the baseline expression of hematopoietic niche regulatory genes in AML-BMSC and HD-BMSC populations. KIT ligand (KITLG), vascular cell adhesion molecule 1 (VCAM1), angiopoietin 1 (ANGPT1), CXCL12, and Jagged1 (JAG1) expression was analyzed in BMSC derived from AML (n=8, independent donors) compared with HD (n=8 independent donors). Expression levels for each gene compared with the GAPDH housekeeping gene are shown as mean ± standard error of the mean (SEM). (F) Quantitative RT–PCR–based analysis of the baseline expression of transcription factors and lineage-specific genes associated with bone, cartilage, and adipose tissue formation in AML-BMSC and HD-BMSC populations. Osterix (SP7), runt-related transcription factor 2 (RUNX2), osteocalcin (BGLAP), osteopontin (SPP1), osteonectin (SPARC), type I collagen a2 chain (COL1A2), alkaline phosphatase (ALPL), aggrecan (ACAN), RUNX3, distal-less homeobox 6 (DLX6), SRY-box 9 (SOX9), fatty acid-binding protein 4 (FABP4), and peroxisome proliferator-activated receptor g (PPARG) expression was analyzed in at least four independent donors. Expression levels for each gene compared with the GAPDH housekeeping gene are shown as mean ± SEM. (G) Tri-lineage *in vitro* differentiation of AML-BMSC and HD-BMSC. BMSC were induced to differentiate along osteogenic, adipogenic, and chondrogenic lineage *in vitro* according to standard protocols. After 21 days of osteogenic induction, cells were stained with Alizarin Red for visualization of Ca2+ accumulation (left panel). Adipogenic differentiation capacity was visualized after 21 days of induction by staining lipid droplets with Oil Red O (middle panel). The osteogenic and adipogenic differentiation was further verified by quantitative RT-PCR–based analysis for the expression of lineages markers (for osteogenesis: ALPL and SPP1; for adipogenesis: lipoprotein lipase (LPL), FABP4, and PPARG). GAPDH was used as reference housekeeping gene. Three-dimensional chondrogenic differentiation assay was used for evaluation of chondrogenic differentiation potential (right panel). After 21 days of induction, sections from cartilaginous pellets were stained with hematoxylin/eosin and Toluidine blue (top panels). Presence of chondrocytes within lacunae and of proteoglycans within cartilaginous matrix (Toluidine blue positive) reveal chondrogenic differentiation. Chondrogenesis was prolonged for an additional 2 weeks to obtain hypertrophic cartilage stained by methylene blue (bottom panels). Calcium deposition was stained in black using von Kossa. Scale bars represent 200 mm. For interindividual comparison the two-

sided unpaired Students' *t*-test was employed. Statistical significance was established at P≤0.05. *P≤0.05, **P≤0.01. und: undifferentiated; diff: differentiated; wks: weeks.

The conventional *in vitro* differentiation assays are partially predictive of the *in vivo* physiologic functions of BMSC as these cultures leverage artificial inducing differentiation factors that do not necessarily reflect the intrinsic physiological potential of the cells.[12] Therefore, in order to accurately assess the *in vivo* functional properties of AML-BMSC in a physiologic environment we used two distinct heterotopic transplantation models to assess the osteogenic activity as well as the capacity to establish a complete hematopoietic niche, respectively. The first assay, which allows evaluation of the BMSC differentiation capacity into osteoblasts based on the formation of histologically-provable bone, was performed by implanting AML-BMSC or HD-BMSC loaded on an osteoconductive hydroxyapatite/tricalcium phosphate carrier in subcutaneous tissues of immunocompromised SCID/beige mice.[13] Histological analysis of the transplants harvested at 8 weeks revealed bone deposition in both groups (Figure 2A-B). Immunostaining of sections with an anti-osterix antibody showed the presence in AML-derived ossicles of osterix-expressing osteoprogenitor cells accompanied by osterix-positive osteocytes (Figure 2C, top panels). Immunostaining with an antiosteocalcin antibody, a marker for mature osteoblasts, revealed a virtual absence of positive cells along the bone surfaces in the AML-derived implants and osteocyte immunoreactivity in both AML- and HD-derived implants (Figure 2C, central panels). Moreover, immunostaining with dentin matrix acid phosphoprotein 1 (DMP1), a marker of mature osteocytes, revealed the presence of DMP1-negative osteocytes in AML-BMSC derived grafts, which differed from HD-BMSC transplants (Figure 2C, bottom panels). In addition, histomorphometric analysis displayed a significantly reduced amount of bone tissue in AMLderived implants (bone area/tissue area [B.Ar/T.Ar] %; AML-derived *vs*. HD-derived implants: 3.33±1.11 *vs*. 10.24±1.28, P=0.002)

(Figure 2A, right panel). Moreover, no changes were detected in the mineralized surface covered by multinucleated tartrate-resistant acid phosphatase (TRAP) positive osteoclasts (OcS/MS %) (Figure 2D). Mirroring this histologic finding, gene expression of the osteoclast differentiation regulators RANKL and OPG evaluated by quantitative RT-PCR in basal BMSC from both sources was similar (Figure 2E). These data indicate an impaired osteogenic potential of AML-derived BMSC, suggesting that leukemia cells can interfere with the maturation of osteoblast precursors which in turn results in reduced bone formation in the absence of changes in osteoclastic activity. It cannot be excluded that the osteogenic differentiation blockade may be a result of epigenetic changes in AML-BMSC affecting the genes involved in osteogenic cell development, such as PTX2 and TBX15 transcription factors.7 Our finding is consistent with previous reports demonstrating perturbation of the osteogenic niche in AML mouse models and in AML and myelodysplastic patients.[2,4,14] Specifically, studies have shown that AML leads to the accumulation of osterix-expressing osteoblast-primed cells in murine BM.[15]

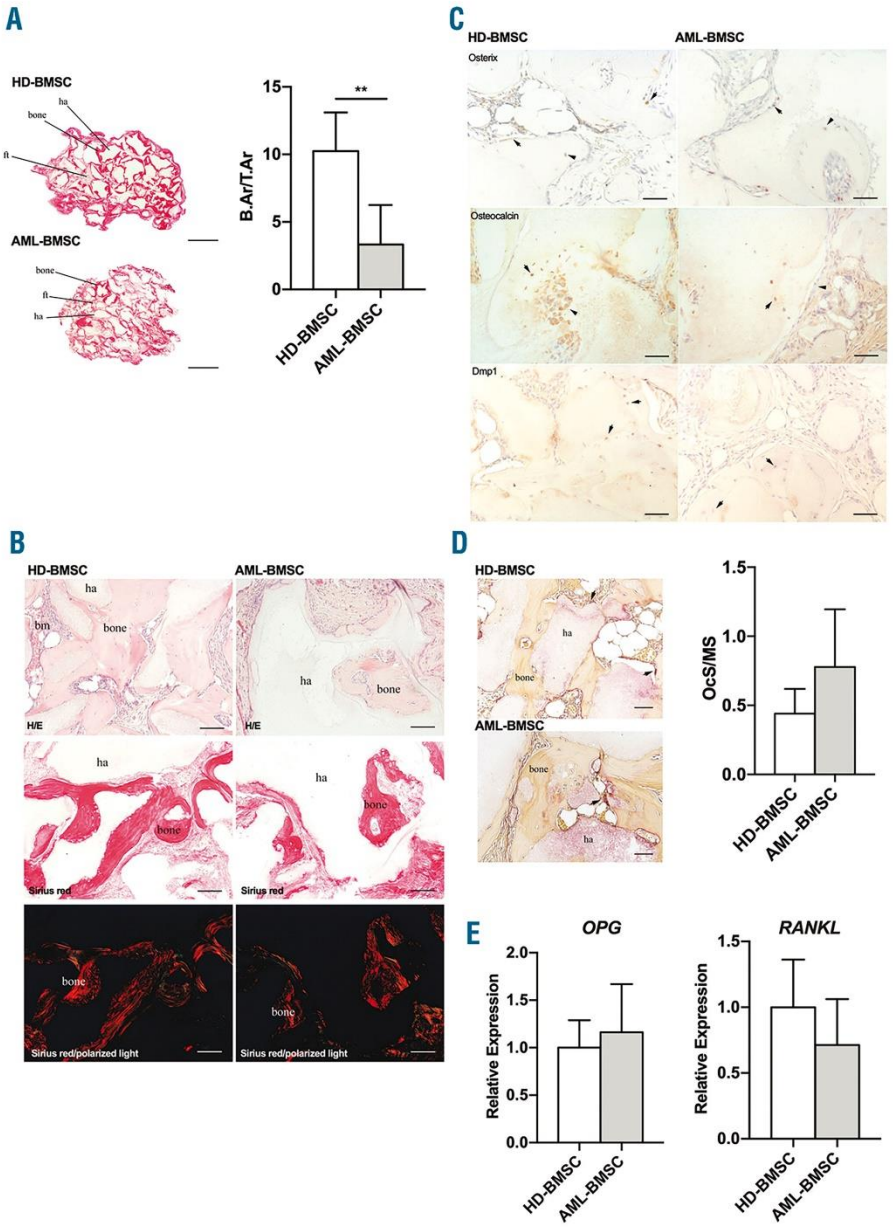

Figure 2. Altered *in vivo* osteogenic potential of acute myeloid leukemia bone marrow stromal cells. (A) In order to study osteogenesis *in vivo* a heterotopic transplantation assay was performed. $2x10^6$ acute myeloid leukemia bone marrow stromal cells (AMLBMSC) or healthy donor BMSC (HD-BMSC) were loaded onto a hydroxyapatite/tricalcium phosphate carrier and transplanted subcutaneously in the back of the immunocompromised SCID/beige mice. After 8 weeks, the grafts were harvested. Whole-mount Sirius red stained

sections of HD-BMSC and AML-BMSC derived grafts (left panel). Scale bars represent 1 mm. Analysis of bone area/tissue area (B.Ar/T.Ar) by histomorphometry (right panel). Columns show the mean ± standard deviation of AML (n=7 grafts from three independent donors) and HD (n=6 grafts from two independent donors). **P≤0.01. (B) Histological analysis of hematoxylin/eosin (H/E) stained sections (top panel) of both AML-BMSC and HD-BMSC heterotopic transplants revealed new bone formation along the hydroxyapatite carrier, and marrow cavities with adipocytes and host hematopoietic cells. Transmitted (central panel) and polarized- (bottom panel) light view of the same microscopic field stained with Sirius red highlighted the newly formed bone and its lamellar structure. Scale bars represent 100 mm. (C) Representative images of immunohistochemical staining for osterix (upper panel), osteocalcin (central panel), and DMP1 (bottom panel). Osterix positive osteoprogenitor cells and osteocytes (arrow and arrowhead, respectively) are detected in AML-derived samples. Conversely, in AML-BMSC derived grafts osteocalcin expression is closely restricted to osteocytes, as opposed to HD-BMSC transplants in which osteocalcin expression is detected either in osteoblasts and osteocytes (arrow and arrowhead, respectively). Moreover, osteocytes in AML-BMSC derived grafts are completely negative for DMP1, contrary to what was observed in HD-derived samples (arrowhead). Scale bars represent 50 mm. (D) Representative images of sections stained for the osteoclastic marker TRAP (left panel). Scale bars represent 50 mm. Quantification of the mineralized surface (bone surface plus scaffold surface) lined by TRAP+ multinucleated osteoclasts (OcS/MS, right panel). Columns show the mean ± standard deviation of AML (n=6 grafts from two independent donors) and HD (n=4 grafts from two independent donors). (E) Quantitative RT-PCR–based analysis of the baseline expression of osteoclasts differentiation regulators osteoprotegerin (OPG) and receptor activator of nuclear factor-κ B ligand (RANKL) in BMSC derived from AML (n=8 independent donors) compared with HD (n=8 independent donors). Expression levels compared with the GAPDH housekeeping gene is shown as mean ± standard error of the mean.

We next assessed the ability of AML-BMSC to form a BM cavity and a functional stromal niche using a model consisting in *in vivo* implantation of cartilage pellets followed by the progressive substitution of cartilage by marrow through a process we named "endochondral myelogenesis".[16] AML-BMSC and HD-BMSC were grown as unmineralized pellets in chondrogenic differentiation medium and then implanted subcutaneously into NSG mice. After 8 weeks, implanted chondroid pellets were replaced by a BM hematopoietic microenvironment composed of human-derived skeletal tissues (bone, cartilage, fat, and perivascular cells), as confirmed by staining with human-specific LaminA/C, and mouse-derived hematopoietic cells (Figure 3A-B). Furthermore, in AMLossicles we detected the presence of human CD146-positive stromal cells associated with the vessel wall (Figure 3B). Interestingly, AML-BMSC derived ossicles contained a significantly increased fraction occupied by adipocytes, then compared to HD-BMSC transplants (adipocyte area/marrow area [Ad.Ar/Ma.Ar] %; AML-derived *vs*. HD-derived implants: 1.92±0.42 *vs*. 0.73±0.22; P=0.037) (Figure 3C). Our results agree with other reports showing that BM-stromal progenitors from AML mice have an increased adipogenic differentiation ability.3 Moreover, Lu et al. found that marrow of AML patients in remission had less adipocyte content than cases from non-remission marrows as compared with diagnostic marrows.[17] Lastly, the amount of hematopoietic tissue and the myeloid/erythroid (MPO+/TER-119+) ratio in normal and patient-derived ossicles were similar (Figure 3D).

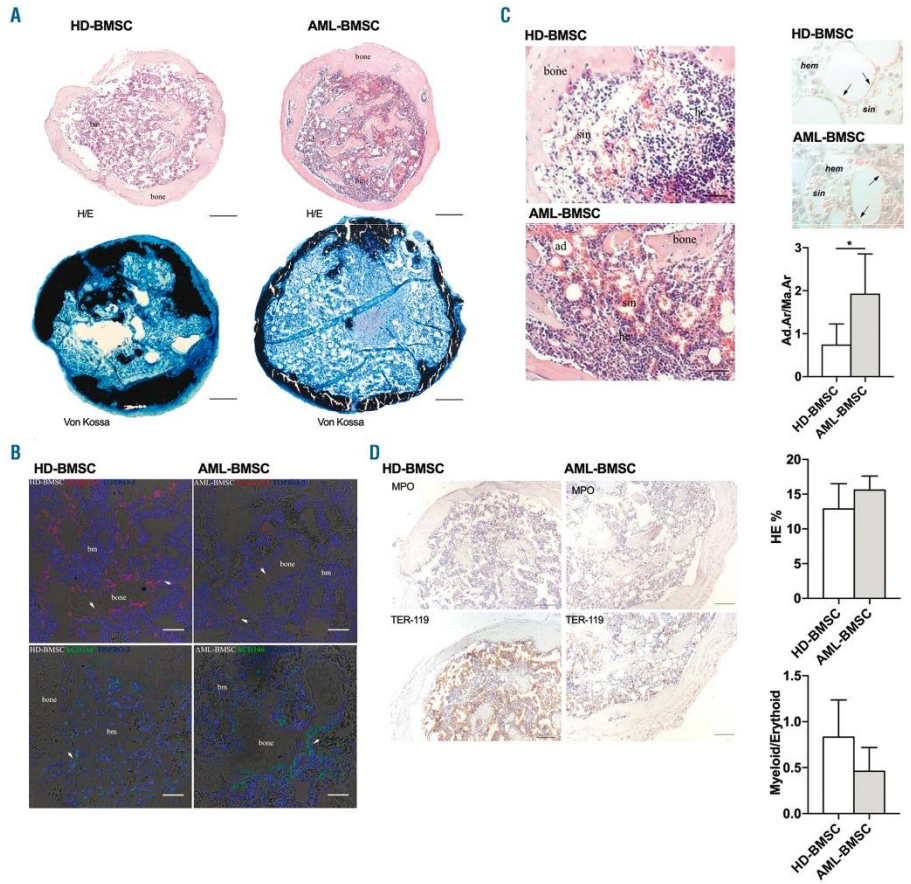

Figure 3. Altered *in vivo* bone marrow stromal niche formation by acute myeloid leukemia bone marrow stromal cells. (A) In order to study the capacity to recapitulate the hemopoietic niche *in vivo,* acute myeloid leukemia bone marrow stromal cells (AML-BMSC) or healthy donor BMSC (HD-BMSC) were cultured for 3 weeks as micro-masses in chondrogenic differentiation medium and then unmineralized pellets were transplanted subcutaneously into immunocompromised NSG mice. Eight weeks after transplantation, the bone/BM organoids were harvested. Representative images of AML- and HD-derived grafts are shown. Hematoxylin/eosin (H/E) stain (upper panel) revealed the presence, in both AML- and HD-ossicles, of a bony cortex with an inner marrow cavity containing murine hematopoietic cells. Undecalcified von Kossa/methylene blue stained sections (bottom panel) evidenced comparable levels of bone mineralization in AML- and HD-derived ossicles. Scale bars represent 200 mm. (B) Confocal fluorescence images confirming the human origin of the heterotopic ossicle. The human specific LaminA/C is detected in the nuclear membrane of osteocytes (arrows) and spread marrow cells (top panel). Bottom panel shows representative images of immunofluorescence staining for hCD146 of BMSC in association with vessels

walls. Cell nuclei were stained with Topro-3 iodide. Scale bars represent 50 µm. (C) Details of marrow tissues in AML- and HD-derived ossicles are shown (H/E stain, left panel). Trabecular bone, hematopoiesis (he), adipocytes (ad) and sinusoids (sin) are observable (scale bars: 50 mm). Adipocytes were stained for perilipin (right top panel). Quantitative analysis of BM fraction occupied by adipocytes within bone/BM organoids (adipocyte area/marrow area [Ad.Ar/Ma.Ar], right low panel) demonstrated that *in vivo* adipogenic differentiation was increased in AML-BMSC. Columns shows the mean ± standard deviation (SD) of AML (n=5 grafts from three independent donors) and HD (n=5 grafts from two independent donor). (D) Quantification and characterization of hematopoietic tissue in the intertrabecular space within the ossicles. Evaluation of the presence of myeloid (MPO+) and erythroid cells (Ter-119+) within murine hematopoietic foci of AML- and HD-ossicles (left panel). Scale bars represent 100 mm. Quantification of total hematopoietic tissue in bone/BM organoids (right top panel). Quantification of myeloid/erythroid ratio in organoids (right low panel). Columns show the mean ± standard deviation of AML (n=4 grafts from three independent donors) and HD (n=2 grafts from two independent donor). bm: bone marrow.

In conclusion, we have demonstrated using *in vivo* physiologic models that AML-BMSC function is significantly altered in mature bone formation and niche composition. As demonstrated by these *in vivo* transplantation assays, BM-stromal progenitors from pediatric AML patients, even when removed from their pathological environment, show an intrinsically abnormal differentiation pattern with altered osteogenesis and increased adipogenic potential, which is not easily detectable by canonical *in vitro* assays. This suggests an instructive role of leukemic cells on the BM microenvironment that can contribute to the generation of a supportive niche for leukemic cells themselves. As our study leveraged *in vivo* models that appropriately reproduce the human BM niche, our data may have an important clinical relevance. Understanding the unique characteristics of the AML osteogenic niche represents a critical step towards unraveling the mechanisms underlying osteogenic niche-mediated support of AML cells and leukemic progression. Our data suggests the possibility to target stage-specific cells of the osteogenic lineage to normalize the hostile BM niche and

suppress AML cell development and proliferation with an ultimate goal of inducing deep remissions and controlling long-term disease.

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Supplementary Appendix

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-PaqueTM 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 (BMNICHE 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 × 106 cells/cm2 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 (CFUF) assay. Briefly, BMSC from passage 0 were seeded at clonal density (1.6 cells/cm2) 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/cm2) 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 (SigmaAldrich). 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/cm2) with osteogenic induction medium consisting of DMEM-low glucose (Gibco), supplemented with 10% FBS (Biosera), 100 nmol/L dexamethasone (Sigma-Aldrich), 10 mM ßglycerol-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 ITSTM 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-PrkdcSCIDLystbg/Crl) or NSG (NOD.Cg-PrkdcscidIl2rgtm1Wjl/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 2x106 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-methylmethacrylate (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 antimouse 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.

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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

CHAPTER 4: AML ALTERS BONE MARROW MESENCHYMAL STROMAL CELL OSTEOGENIC COMMITMENT VIA NOTCH SIGNALING

Manuscript in preparation

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ABSTRACT

Acute myeloid leukemia (AML) is a hematological malignancy characterized by the accumulation of immature myeloid blasts in the bone marrow (BM). This abnormal growth of AML cells disrupts normal hematopoiesis and alters the BM microenvironment components to establish a niche supportive of leukemogenesis. Among the latter, bone marrow mesenchymal stromal cells (BMSCs) play a pivotal role in giving rise to essential elements of the BM niche, including adipocytes and osteoblasts. Several animal models have shown that bone tissue was hijacked by AML cells, which skew BMSCs toward an ineffective osteolineage differentiation with an accumulation of osteoprogenitors in the AML BM. However, little is known about the mechanisms by which AML cells affect osteogenesis.

We studied the effect of AML cells on the osteogenic commitment of normal BMSCs. Using a 2D co-culture system, we found that AML cell lines and primary blasts, but not normal CD34+ cells, induce in BMSCs an ineffective osteogenic commitment, with an increase of the early-osteogenic marker TNAP in the absence of the late-osteogenic gene up-regulation. Moreover, the direct interaction of AML cells and BMSCs is indispensable in influencing their osteogenic differentiation. Mechanistic studies identified a role for AML-mediated Notch activation in BMSCs contributing to their ineffective osteogenic commitment. Inhibition of Notch using a γ-secretase inhibitor strongly influenced Notch signaling in BMSCs and abrogated the AML-induced TNAP up-regulation. Furthermore, preliminary results using an *in vivo* heterotopic subcutaneous osteogenesis transplantation assay seem to confirm that BMSCs showed a decrease or their capacity to form mineralized bone when implants were performed in AML xenograft mice. This seems to be ascribable to an important reduction of osteoblast maturation in the absence of changes in osteoclastic activity.

Together, our data support the hypothesis that AML infiltration induces a leukemia-supportive preosteoblast-riche niche in the BM, which can be partially ascribed to AML-induced activation of Notch signaling in BMSCs.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous clonal hematopoietic malignancy characterized by the proliferation and accumulation of transformed immature myeloid precursors in bone marrow (BM) which, competing with hematopoietic stem cells (HSC) for nourishment and niche occupancy, lead to impaired production of normal blood cells[1]. Murine models of AML show that progressive leukemia infiltration alters the BM niche to support malignancy at the expense of healthy hematopoiesis[2,3].

One of the key components of the BM niche is represented by bone marrow mesenchymal stromal cells (BMSCs) that thanks to their multipotency can differentiate toward different BM niche stromal cells, including adipocytes and osteoblasts. Increasing evidence suggests that BMSCs regulate various biological processes in AML, including metabolic changes and alterations in the expression of pro-survival factors, thereby contributing to drug resistance[4–6]. Notably, the communication between AML cells and BMSCs is bi-directional, also resulting in transcriptional and functional changes in the latter[7,8]. AML-associated BMSCs demonstrated substantial differences in their methylation patterns and a compromised ability in supporting hematopoiesis as compared to their normal counterparts[9,10].

Several murine AML models revealed alterations in BM architecture, with loss of mature osteoblasts and accumulation of osteoprogenitors[7,11,12]. Furthermore, we previously showed that AML patient derived-BMSCs even once removed from their pathological environment, exhibit *in vivo* reduced osteogenic differentiation capability and generate an osteoprogenitors-enriched BM niche[13]. However, little is known about AML BM architecture in humans and how findings from murine models can be relevant to human disease. Moreover, it is not clear whether changes in the BM microenvironment are induced directly by AML cell infiltration or indirectly through other factors.

Recent studies have focused on the role of Notch and Wnt signaling in the context of crosstalk between AML cells and BMSCs[14–16]. Notch signaling is involved in BMSC-mediated

chemoresistance and support of AML cell growth[14,15]. Moreover, Notch signaling alterations in BM stromal cells have been correlated with a pathogenic role in AML[17].

Notch signaling plays a key role also in the regulation of BMSC differentiation as well as in osteoblastogenesis and bone homeostasis[18], and enhancement of its activation in BMSCs has already been related to an accumulation of immature preosteoblasts[19].

In this study, we analyzed, using *in vitro* and *in vivo* systems, the effect of AML cells on the osteogenic differentiation potential of BMSCs. BMSCs exposed to AML cells acquired defects in osteogenesis, with altered expression of osteogenic markers *in vitro* and reduced bone formation *in vivo*. This ineffective osteogenic commitment seems to be related to AML-mediated activation of Notch pathway on BMSCs.

MATERIAL AND METHODS

Cell lines and AML primary cells

KG-1, THP-1, U-937, HL-60 (AML cell lines) and NALM-6 and 697 (ALL cell lines) have been obtained from the American Type Culture Collection (ATCC). The KG-1 and U-937 cell lines were maintained in culture with RPMI 1640 medium (EuroClone, Milan, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (EuroClone), 25 IU/ml of penicillin and 25 mg/ml of streptomycin (EuroClone). The THP-1, NALM-6, and HL-60 cell lines were maintained in culture with 10% FBS Advanced RPMI 1640 complete medium (GibcoTM, Thermo Fisher Scientific, Waltham, USA). The 697 cell line was maintained in culture with 20% FBS Advanced RPMI 1640 complete medium.

Primary AML cells were obtained from peripheral blood (PB) or BM samples collected from newly diagnosed AML patients, after obtaining informed consent. Briefly, PB- or BM-mononuclear cells (MNCs) were isolated using a Ficoll-PaqueTM Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient separation. Clinical and biological AML patients' features are reported in Table 1.

AML SAMPLES	AGE (years)	Sex (F/M)	BLASTS (%)	FAB	SOURCE (BM/PB)	KARIOTYPE	MUTATIONS
AML1	75	N/A	90	N/A	BM	N/A	N/A
AML2	11	F	85	M2	BM	46,XX,t(8;21)(q22;q22)[18]/46, XX[2]	N/A
AML3	13	M	70	M4	BM	46,XY,t(9;11)(p21;q23)][25]	N/A
AML4	12	F	90	M3	BM	46,XX[20]	t(15;17)
AML5	74	M	70	M3	BM	46,XY,t(15;17)(q24;q21)[19]/46 ,XY[1]	t(15;17)
AML6	7	F	85	M5	BM	46,XX,t(8;19;21)(q22;?p13;q22)[26]/46,XX[2]	C-KIT+
AML7	64	F	62	M4	BM	43,XX,del(5)(q?31q35),- 7,i(8)(q10),der(?16)t(16;21)(q1 1;q11),-18,-21[20]	N/A
AML8	70	F	90	M1	BM	46,XX[20]	FLT3-ITD
AML9	71	F	70	M1-2	BM	N/A	FLT3-ITD
AML10	68	M	70	N/A	BM	46,XY[20]	NPM1 MUT
AML11	35	M	80	M1	BM	46,XY[20]	FLT3-ITD
AML12	6	F	70	M5	BM	46,XX[20]	MLL-RIAR (11q23)
AML13	73	M	80	M1	BM	42-44,X,- Y,?del(4)(q12),add(5)(p1?5),- 7,del(7)(q?31),-8, add(9p2?1), i(12)(q10),+?21,+mar,inc[cp20]	N/A
AML14	4	M	90	M3	BM	46,XY,t(15;17)(q24;q21)[15]/46 ,XY[5]	N/A
AML15	48	M	70	N/A	PB	N/A	N/A
AML16	59	F	80	M1	BM	46,XX[17]	N/A
AML17	7	M	80	M3	BM	N/A	t(15;17)
AML18	62	M	85	M0	BM	46,XY,-7,+MAR[15]/46,XY[7]	N/A
AML19	53	M	80	M1	BM	46,XY,inv(16)(p13q22[19]/46,X Y,[1]	N/A
AML20	11m	M	80	M5	PB	46,XY,t(9;11)(p21;q23)[20]	N/A
AML21	75	M	70	M0	BM	46,XY[20]	N/A
AML22	3	M	80	M5A	PB	45,XY,der(13;14)(q10;q10)[20]	FLT3-ITD
AML23	92	M	70	M5	BM	46,XY[25]	N/A
AML24	79	M	90	M4	BM	46,XY[25]	N/A
AML25	62	M	80	M4e	BM	46,XY,inv(16)(p13q22)[19]/46, XY[1]	N/A
AML26	72	F	95	M1	BM	N/A	FLT3 MUT
AML27	1	M	75	M5A	PB	47,XY,+1,i(1)(q10)[20]	FLT3 (D835Y)
AML28	19	M	90	N/A	BM	46,XY[20]	N/A

AML29	44	M	90	M0	BM	46,XY[20]	NPM1 MUT
AML30	15	F	80	M4	BM	46,XX,t(8;21)(q22;q22),?del(9)(q?12q?22)[19]/46,XX[1]	AML1-ETO
AML31	17	M	85	M3	BM	N/A	t(15;17)
AML32	80	M	60	M2	BM	51,XY,+8,+9,+11,+13,+14[16]46,XY[4]	N/A
AML33	76	F	95	N/A	BM	46,XX[20]	N/A
AML34	60	F	87	M0	PB	42-44,XX,add(9)(p2?3),-5,-12,-13.-17,+4mar,inc[cp24]	N/A
AML35	54	F	90	M4	BM	47~49.XX,+8[13],+10[14],+19[21][cp21]	N/A

Table1. Clinical and biological patients' details.

Isolation and culture of BMSCs

BMSCs were isolated from the BM of 24 healthy donors undergoing BM harvest for allogeneic transplantation. Each BM sample was subjected to centrifugation over a Ficoll-Paque gradient to separate MNCs. BM-MNCs were seeded at a density of 2×10^5 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM)–low glucose (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 25 IU/ml of penicillin and 25 mg/ml of streptomycin. Non-adherent cells were removed 24-48 hours after initial plating by washing with phosphate-buffered saline (PBS, Euroclone). The cultures were maintained in the complete medium until they reached 70-80% confluence and then detached with 0.05% Trypsin-EDTA (Euroclone) and re-seeded at $2x10^3$ cells/cm^2. BMSCs cultures were characterized by assessing morphology, clonogenic potential, proliferation, immunophenotype, and tri-lineage differentiation potential (adipogenic, osteogenic, and chondrogenic differentiation), as described in Pievani et al 2021[13], and used for our experiments until passage 7.

CD34+ progenitor isolation

Human CD34+ progenitors were isolated from cord blood (CB) units, after having obtained informed consent. Briefly, CB-MNCs were isolated by density gradient centrifugation, followed by

immunomagnetic selection using the CD34 MicroBeads kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) coupled with MACS LS Columns and MidiMACS Separator (all Miltenyi Biotec), according to the manufacturer's instructions. Purity has been determined by flow cytometry using anti-CD34 PECy7 (clone 8G12; BD Biosciences Franklin Lakes, NJ, USA). The median purity of isolated CD34+ cells was 96.1% (range from 92.5% to 99.4%).

Induction of osteogenesis

BMSCs ($2x10^4$ cells/cm^2) were cultured in the osteogenic medium consisting of DMEM-low glucose, supplemented with 10% FBS, 0.1µM dexamethasone (Sigma-Aldrich, Missouri, USA), 10mM beta-glycerophosphate (Sigma-Aldrich), and 0.05 mM L-ascorbic acid (Sigma-Aldrich). Cell cultures were maintained for different times, as indicated in the manuscript.

Co-culture experiments

BMSCs were seeded at $2x10^4$ cells/cm^2 in complete DMEM in a 12-well plate (Corning, NY, USA) or a 6-well plate (Corning). After reaching 70-80% confluence, typically in 24 hours, leukemia cells were added. For AML and ALL cell lines, we plated $7x10^5$ cells/well in a 12-well plate and $2x10^6$ cells/well in a 6-well plate in basal medium (complete DMEM) or osteogenic medium. For primary AML cells or healthy CD34+ progenitors, we plated $1.4x10^6$ cells/well in a 12-well plate. The times of different co-culture experiments are indicated in the text.

For purification of BMSCs after co-culture, the adherent layer was dissociated using 0.05% Trypsin-EDTA after washing with PBS to remove non-adherent cells. Cells were magnetically separated using anti-human CD90 MicroBeads and MidiMACS Separator (all Miltenyi Biotec), according to the manufacturer's instructions. The positive fraction was resuspended in the QIAZOL reagent (QIAGEN Inc., Valencia, CA).

To evaluate the effect of cell-cell contact, the co-cultures were performed using a transwell system (0.4 um pores polyester

membrane, Corning, NY, USA) with BMSCs in the bottom well and AML cells in the insert.

To evaluate the effects of Notch activation, BMSCs were stimulated with immobilized Recombinant Jagged1 Fc Chimera (JAG1 5µg/ml, R&D Systems, Minneapolis, MN, USA). Notch activation was carried out for 72 hours. In some experiments, DAPT (20 µM) was added to the culture medium.

To inhibit Notch pathway, DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-s-phenylglycinet-butylester; Sigma-Aldrich) was added to the medium with a final concentration of 20µM. Vehicle (DMSO) was added in the control group.

Flow cytometry

Multiparameter analyses of stained cell suspensions were performed on FACS CANTO II (BD Bioscience) and analyzed with FlowJo-v10.8.1 software (Treestar, Ashland, OR, USA).

To quantify the level of TNAP in BMSCs after co-culture with AML or ALL cell lines, primary AML cells, or healthy CD34+ progenitors, the adherent layer was subjected to trypsinization after washing with PBS to remove non-adherent cells. Cells were stained with TNAP-PE (clone W8B2, Biolegend, San Diego, CA, USA) and with a fluorochrome-conjugated mAb specific to exclude residual leukemia cells. Specifically, CD33-PE-Cy7-A (clone P67.6; BD Bioscience) was used to gate AML cells, CD19-Pecy7 (J3-119, Beckman coulter) was used to gate B-ALL cells and CD34-FITC (clone 581; BD Biosciences) was used to gate CD34+ progenitors.

The following antibodies were used for the analysis of BMSCs: CD90-PE (clone 5E10; eBioscience, San Diego, CA, USA), CD73-PE (clone AD2; BD Biosciences), CD105-PE (clone SN6; eBioscience), and CD146-PE (clone P1H12; BD Biosciences).

Median fluorescence intensities were calculated in co-cultured BMSCs in comparison to control with FlowJo software.

Gene expression analysis by quantitative real-time RT-PCR

RNA was extracted from cell cultures with QIAZOL reagent (Qiagen) according to manufacturer's instructions. RNA was extracted from cell lines or from AML diagnostic samples with Trizol reagent (Applied Biosystem) following manufacturer's instructions. 1 µg of total RNA was retro-transcribed using the SuperScript II Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific), in the presence of random hexamers (Invitrogen). Real-time PCR was performed in an ABI 7900 real-time PCR system (Applied Biosystems), using TaqMan Gene Expression Master Mix and TaqMan probes reported in Table 2 (Roche Diagnostics, Rotkreuz, Switzerland). Data were normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. The relative mRNA expression was calculated by the comparative threshold cycle (Ct) method, using the formula $2^{-\Delta\Delta Ct}$. The results are expressed as $2^{-\Delta Ct}$ or fold increase as indicated in bar graphs.

	Protein	Gene Symbol	Primer for RT-PCR (TaqMan assay number)
Housekeeping	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	4333764F
Osteogenic Genes	Osteopontin	SPP1	Hs00959010_m1
	Alkaline Phosphatase	ALPL	Hs01029144_m1
	Osteoclacin	BGLAP	Hs00609452-g1
	Runt-related transcription factor 2	RUNX2	Hs00231692_m1
	Osterix	SP7	Hs01866874_s1
Osteoclast Genes	Receptor activator of nuclear factor-kappaB ligand	RANKL	Hs00243519_m1
	Osteoprotegerin	OPG	Hs00171068_m1
Hematopoiesis-support Genes	Vascular cell adhesion molecule 1	VCAM1	Hs01003372_m1
	Angiopoietin 1	ANGPT1	Hs00181613_m1
	Bone morphogenic protein 4	BMP4	Hs00370078_m1
Leukemogenesis-support Genes	C-C Motif Chemokine Ligand 2	CCL2	Hs00234140_m1
	C-X-C motif chemokine ligand 8	CXCL8	Hs00174103_m1
	Interleukin-6	IL6	Hs00174131_m1
Notch Signaling Genes	Jagged1	JAG1	Hs00164982_m1
	Jagged2	JAG2	Hs00171432_m1
	Delta Like Canonical Notch Ligand 1	DLL1	Hs00194509_m1
	Notch Receptor 1	NOTCH1	Hs01062014_m1
	Notch Receptor 2	NOTCH2	Hs01050719_m1
	Notch Receptor 3	NOTCH3	Hs01128541_m1
	Hes Family BHLH Transcription factor 1	HES1	Hs01172878_m1
	Hes related Family BHLH Transcription Factor with YRPW Motif 1	HEY1	Hs00232618_m1

Table 2. quantitative real-time RT-PCR primers

In vivo heterotopic subcutaneous osteogenesis transplantation assay

In vivo experiments were performed in 8- to 10-week-old NSG (NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ) mice from Charles River Laboratories. All animal procedures were performed under license approved by the Italian Ministry of Health and conformed with national and international law and policies. Transplantation of BMSCs on hydroxyapatite/tricalcium phosphate (HA/TCP) carrier was performed as previously reported[22]. Briefly, for each transplant $2x10^6$ cells were loaded on 40 mg of HA/TCP. Cell/carrier constructs were then transplanted subcutaneously into NSG mice, previously anesthetized with an intramuscular injection of Zoletil 20 (Virbac; 5 mL/g of body weight). After 5 weeks from the implant, animals were intravenously infused with the HL-60 AML cell line ($1x10^4$ cells/mouse). Animals were euthanized after 8 weeks and implants were harvested for histological analyses.

Histology and Histomorphometry

Obtained implants were fixed with 4% formaldehyde in PBS pH 7.4, decalcified in 10% EDTA, and routinely processed for paraffin embedding. Four µm thick paraffin sections were stained with Hematoxylin and Eosin (H/E) or Sirius Red and analyzed by light microscopy. 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 manufacturer's instructions. Transmitted 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. 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) and Osteoclast Surface/Mineralized Surface (OcS/MS) according to the

guidelines of the American Society of Bone and Mineral Research[20].

Immunolocalization

Immunolocalization of Osterix was performed using a rabbit polyclonal anti-human SP7 antibody (1:100; ab22552, Abcam) with an overnight incubation at +4°C. 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).

Statistical analysis

Results are shown as means ± SEM. Pairwise comparisons were performed by means of *t*-test, paired or not, depending on the experimental context. The tests were performed two-sided and the significance level was set as 0.05. Graphs and statistical analyses were produced with the GraphPad Prism 7.0 Software version (GraphPad Software Inc).

RESULTS

AML cells prime normal BMSCs toward osteoprogenitors by direct cell-cell contact

We previously demonstrated that BMSCs derived from pediatric AML patients present a significant impairment in mature bone formation *in vivo*[13]. To try to understand if alterations of BMSC osteogenic commitment can be specifically caused by AML infiltration on healthy BM, we evaluated the expression of TNAP, an early osteogenic marker upregulated on osteoblast-primed AML derived-BMSCs[21], on normal BMSCs co-cultured with different AML cell lines (HL-60, KG-1, THP-1, U-937) in an *in vitro* 2D system, as described in Figure 1A. After 24, 48, and 72 hours of co-culture, TNAP expression on the cell surface of BMSCs was assessed by flow cytometry. AML cells, differently from ALL cells, induced on BMSCs a progressive increase of TNAP expression, which reached significance after 72 hours (Supplementary Fig. 1A). Notably, other BMSC-specific surface markers were not altered by exposure to AML cells (Supplementary Fig. 1B). After 72 hours of co-culture with all the tested AML cell lines, TNAP resulted up-regulated in BMSCs both in basal (Relative TNAP expression of co-cultured *vs* alone BMSCs: + HL-60, $p=0.001$; + KG-1, $p=0.0028$; + THP-1, $p=0.032$; + U-937, $p=0.0178$) and even more in osteoinductive conditions (Relative TNAP expression of co-cultured *vs* alone BMSCs: + HL-60, $p=0.0375$; + KG-1, $p=0.0138$; + THP-1, $p=0.0377$) (Fig. 1B and C). As expected, the osteogenic culture conditions increased *per se* the level of TNAP on BMSCs ($p<0.0001$). Conversely, ALL cell lines did not affect TNAP expression on BMSCs, neither in basal nor in osteo-inductive conditions (Fig.1B and C).

To understand if TNAP up-regulation on BMSCs mediated by AML depends on cell-cell contact or soluble factors, we used a transwell co-culture system. Of note, only AML in direct cell contact increased TNAP expression on BMSCs (direct contact *vs* transwell in basal conditions: HL-60, $p=0.0023$; KG-1, $p=0.0153$; THP-1, $p=0.01$; direct contact *vs* transwell in osteoinductive

conditions: HL-60, p=0.0177; KG-1, p=0.0265; THP-1, p=0.0373) (Fig.1D and E). Similarly, also the AML-conditioned medium did not affect TNAP expression on BMSCs, confirming the importance of cell-cell interaction (data not shown).

Furthermore, we evaluated if also primary blasts from AML patients at the onset can prime BMSCs to osteogenic differentiation. Interestingly, co-culture with primary AML cells lead to a significant up-regulation of TNAP expression in BMSCs (p=0.0011) (Fig.1F). Of note, only 7 of 32 (22%) different AML samples tested did not cause changes in TNAP expression on BMSCs. No correlation was observed between TNAP up-regulation and genetic or morphologic-specific AML subtype. On the contrary, co-culture with CB-derived CD34+ cells did not affect TNAP surface levels in BMSCs.

These data demonstrate that AML cells prime osteogenic commitment in BMSCs, as highlighted by the upregulation of the early-osteogenic marker TNAP, in a contact-dependent manner.

Figure 1

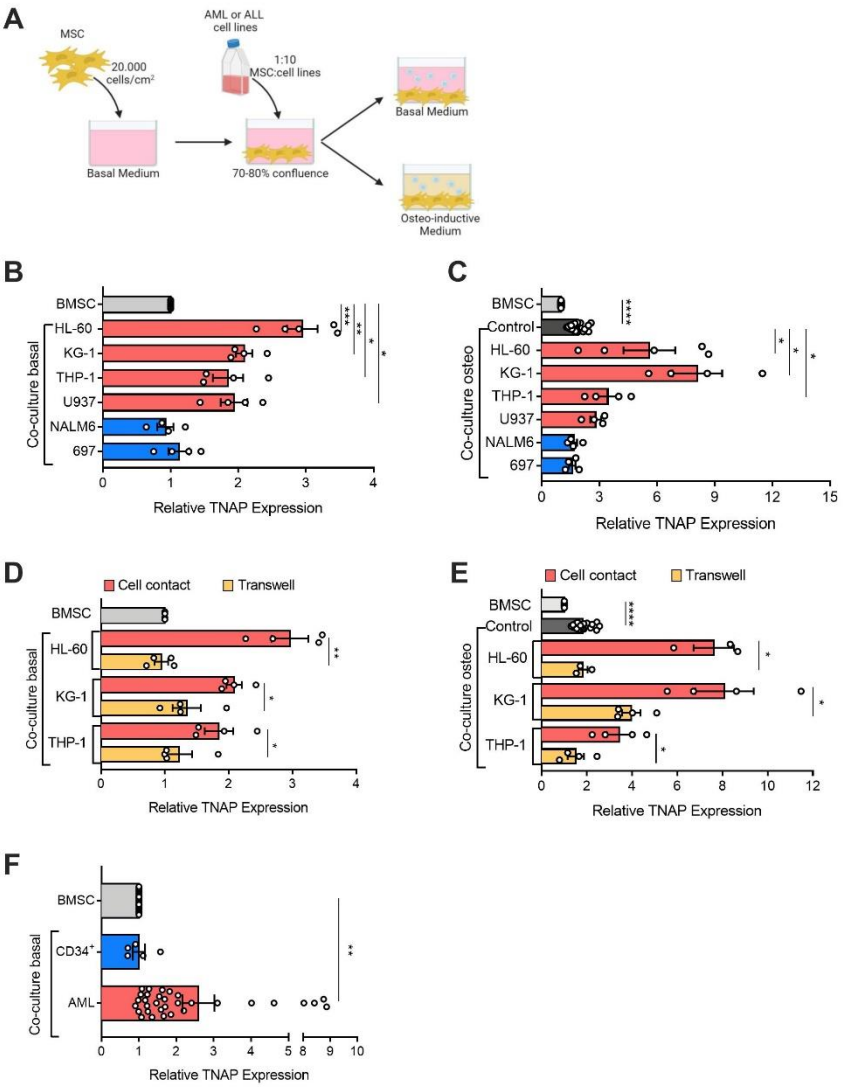

Figure 1. AML cells induce TNAP over-expression on BMSCs in a contact-dependent manner (A) Schematic representation of *in vitro* 2D co-culture system. $7x10^4$ BMSCs were cultured in 12-well plates with AML (HL-60, KG-1, THP-1, U-937) or ALL (697, NALM-6) cell lines in basal or osteo-inductive medium at a ratio of 1:10 (BMSCs:cell line) for 72 hours. (B)-(C) Relative TNAP surface expression on BMSCs assessed by flow cytometry analysis after co-culture in basal (B) or osteo-inductive (C) conditions. For HL-60: n=5

independent experiments using BMSCs from 5 different donors; for THP-1, KG-1, U-937, 697, and NALM-6: n=4 independent experiments using BMSCs from 6 different donors. (D)-(E) Relative TNAP surface expression on BMSCs after co-culture with AML cell lines, in direct-contact or transwell, in basal (D) or osteo-inductive (E) conditions. For THP-1 and KG-1: n=4 independent experiments using BMSCs from 4 different donors; for HL-60: n=3 independent experiments using BMSCs from 3 different donors. (F) Relative TNAP surface expression on BMSCs after co-culture for 72 hours with AML primary blasts or CB-derived CD34+ cells in basal conditions at a ratio of 1:20 (BMSCs:cells). For CD34+: n= 5 independent experiments using 3 different BMSC strains and CD34+ cells isolated from 5 different CB units; for AML blasts: n=32 independent experiments using 10 different BMSC lines and AML blasts from 32 different patients. Data are presented as individual values and the mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, by paired *t*-test.

AML cells inhibit the osteoblast lineage differentiation potential of BMSCs and alter their capacity to support normal hematopoiesis

To deeply investigated AML-induced alterations in the osteogenic differentiation of BMSCs, we evaluated expression changes of osteogenesis-related genes in BMSCs co-cultured with HL-60 AML cell line at 3, 7, 14, or 21 days in basal and osteogenic conditions. Hence, we quantified mRNA levels of early-osteogenic markers (alkaline phosphatase/ALPL, osterix/SP7, and RUNX Family Transcription Factor 2/RUNX2) and late-osteogenic markers (osteopontin/SPP1 and osteocalcin/BGLAP). As expected, we found that ALPL levels were significantly up-regulated after 3 days in the presence of HL-60 cells in basal ($p=0.0207$) and osteogenic conditions ($p=0.035$), confirming the results previously obtained by flow cytometric analysis (Fig.2A). Interestingly, also SP7 expression was transiently up-regulated after 3 days of co-culture, even if this trend did not reach statistical significance. Conversely, RUNX2, another gene involved in the early stages of osteogenic induction, resulted down-regulated since day 3 of co-culture in basal conditions (RUNX2 at 3 days $p=0.0006$; RUNX2 at 7 days, $p=0.0046$), showing a similar trend also in osteogenic ones (Fig. 2A). Late-osteogenic genes osteocalcin and osteopontin were significantly down-regulated in BMSCs co-cultured with HL-60 for

14 and 21 days (in osteogenic conditions: BGLAP at 21 days, p=0.0404; SPP1 at 14 days, p=0.0031; SPP1 at 21 days, p=0.0006), suggesting an impairment in the capacity of BMSCs to differentiate into mature osteoblasts in the presence of AML cells (Fig2B).

As bone remodeling depends not only on osteogenesis but also on bone resorption, we investigated the osteoclastogenic capacity of BMSCs exposed to AML cells, evaluating the expression of osteoclastogenesis regulators Rank-ligand/RANKL and osteoprotegerin/OPG. HL-60 cells reduced the expression of RANKL and OPG on BMSCs and their preosteoblast progeny (at 7 days in basal conditions: RANKL, p=0.0016; OPG, p=0.0101; at 7 days in osteogenic conditions: RANKL, p=0.0076; OPG, p=0.0302). However, the RANKL/OPG index, which is an index of osteoclastogenesis stimulation capacity, was not affected (Fig.2C). These data suggest that AML cells block osteoblast lineage differentiation of BMSCs at a preosteoblast stage.

Figure 2

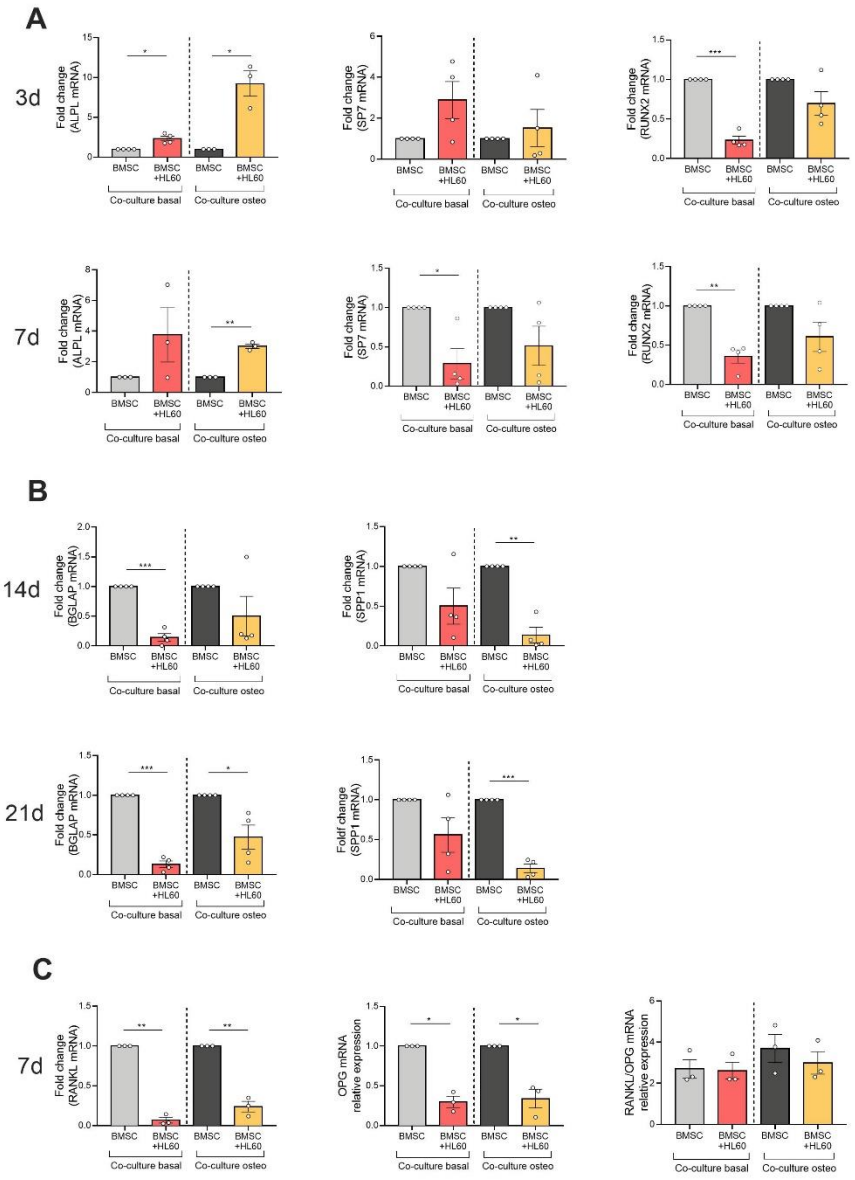

Figure 2. AML cell line HL-60 alters osteoblast-related gene expression in BMSCs during differentiation. (A-B-C) qRT-PCR analysis of early- (ALPL, SP7, and RUNX2) and late-osteogenic (BGLAP and SPP1) factors, and osteoclastogenesis regulators (RANKL and OPG) performed on BMSCs alone or co-cultured with HL-60, in basal or osteogenic conditions at indicated

time points. For early- and late-osteogenic genes (A and B): n=4 independent experiments using BMSCs from 4 different donors; for osteoclastogenesis-related genes (C): n=3 independent experiments using BMSCs from 3 different donors. Data are expressed as fold change respect to control and presented as individual values and mean ± SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, by paired *t* test.

Next, as it was reported that preosteoblasts can favor leukemic cells at the expense of normal hematopoiesis, we focused on AML-induced changes in expression levels of hematopoietic-related genes. HSC-regulating genes (BMP4, ANGPT1, and VCAM1)[22–24] were strongly down-regulated in BMSCs (BMP4, $p<0.0001$; ANGPT1, $p<0.0001$; VCAM1, $p<0.0001$) and their preosteoblast progeny (BMP4, $p=0.0005$; ANGPT1, $p<0.0001$; VCAM1, $p=0.0002$) after exposure to HL-60 AML cells (Fig.3A). On the contrast, genes implicated in leukemogenesis (IL-6, CCL2, and CXCL8)[25–27] were significantly increased in AML-exposed BMSCs in both basal (IL-6, $p=0.0055$; CCL2, $p=0.0105$; CXCL8, $p=0.0201$) and osteogenic conditions (IL-6, $p=0.0005$; CCL2, $p=0.001$; CXCL8, $p=0.0205$) (Fig.3B). These findings support the hypothesis that AML cells influence the BMSC capabilities to contribute to the generation of a tumor microenvironment that becomes permissive to leukemia growth and disrupts normal hematopoiesis.

Figure 3

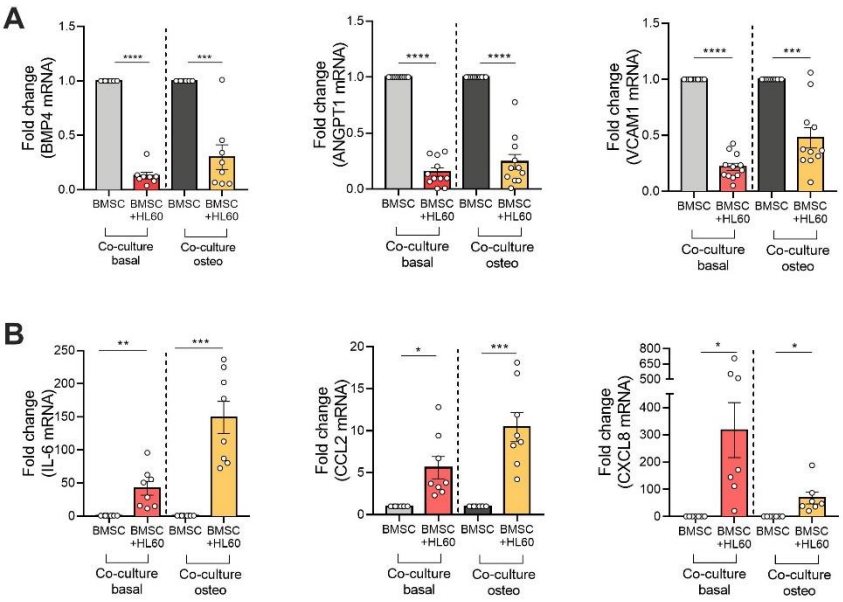

Figure 3 AML cell line HL-60 alters the expression of HSC-regulating and leukemogenesis-supporting genes in BMSCs and their preosteoblast progeny. (A-B) qRT-PCR analysis of HSC-supporting factors (BMP4, ANGPT1, and VCAM1) and leukemogenesis-supporting factors (IL-6, CCL2, and CXCL8) factors performed on BMSCs alone or co-cultured with HL-60, in basal or osteogenic conditions for 72 hours. For ANGPT1 and VCAM1: n=11 independent experiments using BMSCs from 11 different donors. For BMP4, IL-6, and CCL2: n=8 independent experiments using BMSCs from 8 different donors. For CXCL8: n=7 independent experiments using BMSCs from 7 different donors. Data are expressed as fold change respect to control and presented as individual values and mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, by paired *t*-test.

Notch signaling is involved in the crosstalk between AML cells and BMSCs

Given the fact that AML cells interfere in the early stages of BMSC osteolineage differentiation through direct cell contact, we next aimed to identify the underlying mechanisms. As it is known that Notch signaling is involved in the interaction between AML

cells and BMSCs[14,28] and it plays a pivotal role in osteogenic differentiation regulation[19], we explored if Notch signaling relates to the altered osteogenic capacity of BMSCs in presence of AML cells.

We evaluated the expression of main Notch ligands JAG1, JAG2, DLL1, and DLL4 in AML cell lines, AML diagnostic samples, and ALL cell lines. We observed that JAG1 and DLL1 were highly expressed in AML cell lines (Fig.4A). Supported by this evidence, we further confirmed JAG1 and DLL1 expression in diagnostic samples from AML patients, with a wide range of expression (Fig.4B). Moreover, Notch receptors were expressed by BMSCs (Fig.4C).

We then assessed the activity of Notch pathway in BMSCs by measuring mRNA levels of Notch receptors and their transcriptional targets after stimulation with immobilized recombinant Jagged1. Jagged1 stimulation induced a significant increase of NOTCH1 ($p=0.0183$) and NOTCH3 ($p=0.0128$) receptors, as well as of their transcriptional targets HES1 ($p=0.0105$) and HEY1 ($p=0.0169$). Notably, the addition of DAPT, a γ-secretase inhibitor that blocks the generation of Notch intracellular domains, prevented Notch signaling activation (Supplementary Figure 2). We were thus interested in investigating if Notch receptors and their transcriptional targets were also increased in our system of leukemia cell and BMSC co-culture. NOTCH1 ($p=0.0249$) and NOTCH3 ($p=0.0005$) expression was significantly increased in BMSCs co-cultured with HL-60 cells. Further confirmation of Notch activation was raised from the significant increase of mRNA expression of HES1 ($p=0.0044$) and HEY1 ($p=0.0242$) (Fig.4E). Similar results were also found after 72 hours of co-culture with KG-1 AML cell line (Supplementary Figure 3). Our findings thus corroborated the important role of Notch signaling in the crosstalk between AML and BMSCs.

Figure 4

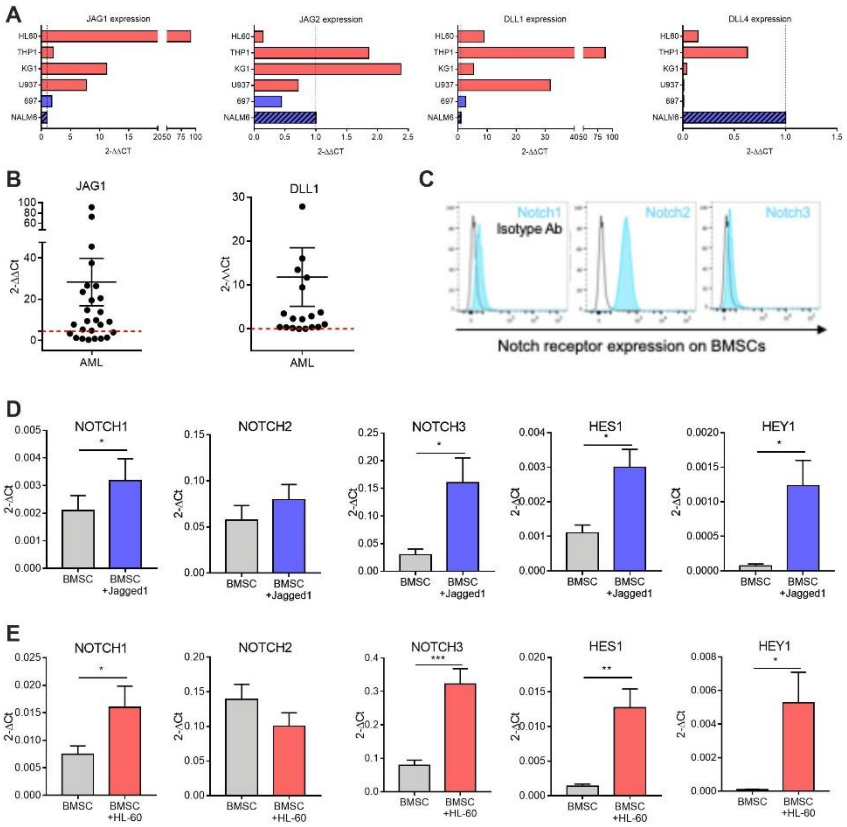

Figure 4. Notch signaling in BMSCs-AML crosstalk. (A) qRT-PCR analysis of JAG1, JAG2, DLL1, and DLL4 in AML cell lines (HL-60, THP-1, KG-1, and U-937) and ALL cell line (697 and NALM-6). On the X axis, the expression is shown as fold change, calculated as $2^{-\Delta\Delta Ct}$ using NALM-6 ALL cell line as reference. (B) qRT-PCR analysis of JAG1 and DLL1 in primary AML blasts. mRNA expression levels are shown as fold change, calculated as $2^{-\Delta\Delta Ct}$ using NALM-6 ALL cell line as reference. Each dot represents a single patient (n=26 for JAG1, n=19 for DLL1). Red dotted lines represent the median value from healthy BM. Mean and SEM were reported. (C) Representative histograms showing the basal expression of Notch receptors on the surface of BMSCs evaluated by flow cytometry. (D) qRT-PCR analysis of mRNA expression of NOTCH1, NOTCH2, NOTCH3 and their transcriptional targets HES1 and HEY1 on BMSCs stimulated with immobilized recombinant Jagged1 for 72 hours. Data are expressed as 2^{-DCt} and presented as mean ± SEM from 7 independent experiments using BMSCs from 7 different donors. (E) qRT-PCR analysis of mRNA expression of NOTCH1, NOTCH2, NOTCH3 and their transcriptional targets HES1 and HEY1 on BMSCs cultured alone or together

with HL-60 cells for 72 hours. Data are expressed as 2-DCt and presented as mean ± SEM from 8 independent experiments using BMSCs from 8 different donors. ***p < 0.001, **p < 0.01, *p < 0.05, by paired *t*-test.

AML-mediated Notch signaling activation in BMSCs is involved in alterations of osteogenesis

To functionally determine the contribution of Notch signaling in affecting BMSCs osteogenic maturation, we first evaluated TNAP expression on the surface of BMSCs stimulated with immobilized recombinant Jagged1 for 72 hours by flow cytometry. Jagged1 treatment significantly increases the relative TNAP expression levels in both basal (p=0.0089) and osteogenic conditions (p=0.0026) (Fig.5A and B). To prove the association between Notch signaling activation and altered osteolineage differentiation in BMSCs after AML infiltration, we used the γ-secretase inhibitor DAPT. First, we added DAPT during the stimulation of BMSCs with immobilized recombinant Jagged1. Adding DAPT prevented the TNAP up-regulation induced by Jagged1 in both basal (p=0.0088) and osteogenic conditions (p=0.0024) (Fig.5A and B). Strikingly, treatment with DAPT successfully avoided the strong up-regulation of TNAP in BMSCs cocultured with AML cell lines restoring its expression to control level (DAPT *vs* vehicle in basal conditions: HL-60, p=0.0006; KG-1, p=0.0032; THP-1, p=0.0037; DAPT *vs* vehicle in osteoinductive conditions: HL-60, p=0.0305; KG-1, p=0.0164; THP-1, p=0.0155) (Fig.5C and D).
Lastly, we demonstrated that DAPT treatment abrogated the TNAP up-regulation induced on BMSCs by primary AML blasts (p=0.0056) (Fig.5E). Notably, DAPT treatment in each specific co-culture brought TNAP relative expression back to basal expression, except for AML#1, AML#11, and AML#12 in which TNAP expression after DAPT-treatment remained higher compared to control condition but still significantly decreased compared to vehicle condition (Fig.5F). All together, these data suggest the involvement of Notch signaling in the boost toward osteoprogenitor of BMSCs induced by AML cells.

Figure 5

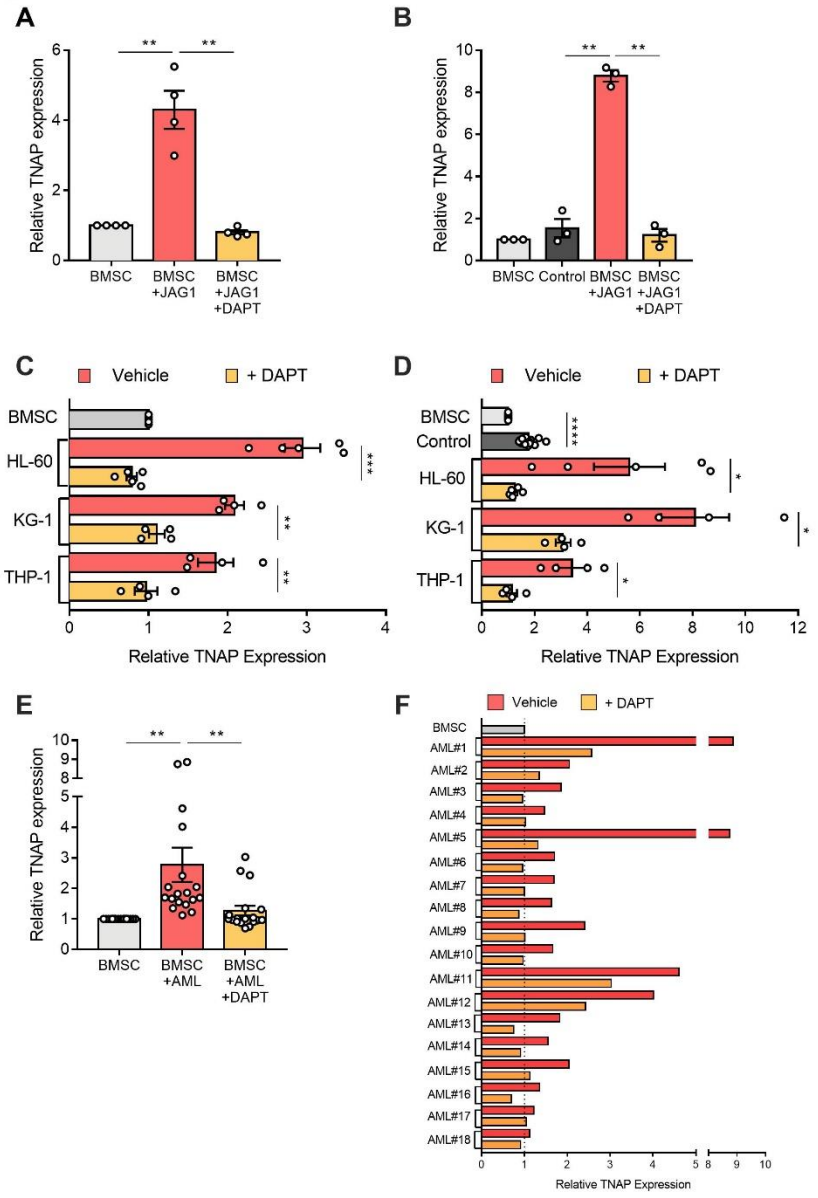

Figure 5. Notch signaling is involved in AML-mediated BMSC osteogenic alteration. (A-B) Relative TNAP surface expression on BMSCs assessed by flow cytometry analysis after 72h of culture in Jagged1-coated plate with or w/o DAPT, in basal or osteo-inductive conditions. For basal conditions (A): n=4 independent experiments using BMSCs from 4 different donors; for osteo-

inductive conditions (B): n=3 independent experiments using BMSCs from 3 different donors. (C-D) Relative TNAP surface expression on BMSCs after 72h of co-culture with AML cell lines (HL-60, KG-1, THP-1) with or w/o DAPT, in basal or osteo-inductive conditions. For both basal (C) and osteo-inductive _(D) conditions: n=5 independent experiments using BMSCs from 5 different donors for HL-60 cell line, n=4 independent experiments using BMSCs from 5 different donors for other cell lines. (E-F) Relative TNAP surface expression on BMSCs after 72h of co-culture with primary AML blasts with or w/o DAPT. n=18 independent experiments using 10 different BMSC lines and AML blasts from 18 different patients. Data are presented as individual values and the mean ± SEM (E) or as individual experiment (F). ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, by paired *t*-test.

Notch signaling activation on BMSCs can trigger an autocrine loop

As BMSCs are reported to express both Notch receptors and ligands, we tested whether the activation of Notch signaling on BMSCs can support an autocrine loop. Therefore, we evaluated the expression of Notch ligands on BMSCs after stimulation. Treatment with immobilized recombinant Jagged1 induced a strong upregulation in the mRNA level of JAG1 on BMSCs ($p=0.0023$) (Fig.6A). Similarly, JAG1 was up-regulated in BMSCs after 72 hours of co-culture with HL-60 AML cell line ($p=0.0018$) (Fig.6B). In this case also DLL1 ($p=0.0009$) and DLL4 ($p=0.0562$) expression resulted significantly increased. This observation suggests that Notch signaling on BMSCs can be activated either as paracrine signal to mediate communication between two different cell types, or as autocrine event.

Figure 6

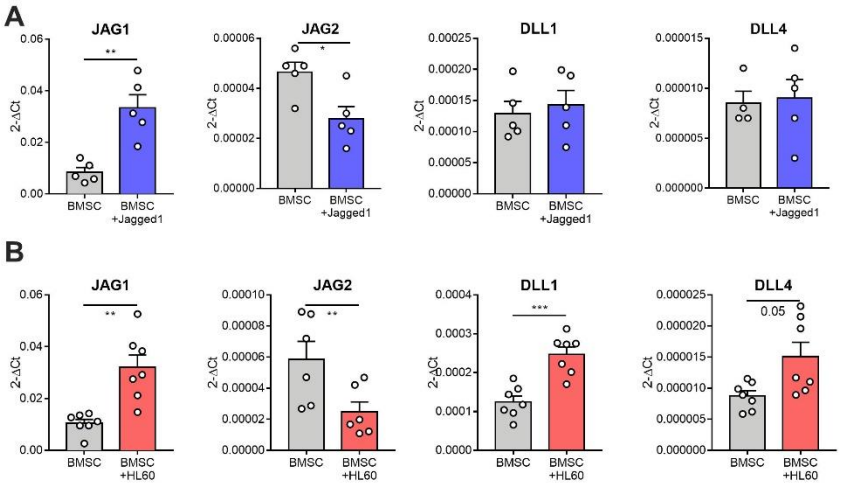

Figure 6. Autocrine loop of Notch signaling in BMSCs(A) qRT-PCR analysis of mRNA expression of Notch ligands JAG1, JAG2, DLL1, DLL4 on BMSCs stimulated with immobilized recombinant Jagged1 for 72 hours. Data are expressed as 2-DCt and presented as mean ± SEM from 5 independent experiments using BMSCs from 5 different donors. (B) qRT-PCR analysis of mRNA expression of Notch ligands JAG1, JAG2, DLL1, DLL4 on BMSCs cultured alone or together with HL-60 cells for 72 hours. Data are expressed as 2-DCt and presented as mean ± SEM from 7 independent experiments using BMSCs from 7 different donors. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, by paired *t*-test.

AML cells inhibit the osteogenic differentiation of normal BMSCs in vivo

To determine if AML cell infiltration can alter the osteogenic commitment of normal BMSCs *in vivo*, we used a heterotopic subcutaneous osteogenesis transplantation assay[29]. In detail, immunocompromised mice were implanted with normal BMSCs loaded on osteoconductive hydroxyapatite/tricalcium phosphate (HA/TCP) carrier and transplanted 5 weeks later with HL-60 AML cells (Fig.7A). Histological analysis of the grafts harvested at 8 weeks revealed bone deposition in both AML xenograft and control mice (not transplanted with AML) (Fig.7B and C).

However, histomorphometric analyses displayed a slightly reduced amount of bone tissue in AML-injected implants (Bone Area/Tissue Area [B.Ar/T.Ar]%, AML-injected *vs* control implants [mean ± SEM]: 4.952 ± 2.474 *vs* 10.36 ± 1.053, p=0.05) (Fig.7C). The decreased bone tissue amount in AML-injected implants was confirmed by the reduction of osteoblast number (number of osteoblasts/bone surface [N.Ob/BS], AML-injected *vs* control implants: 153.9 ± 72.23 *vs* 451.2 ± 160.2) and by the decrease of bone surface covered by osteoblasts (osteoblast surface/bone surface [Ob.S/BS], AML-injected *vs* control implants: 0.013 ± 0.006 *vs* 0.038 ± 0.008) (Fig.7C). On the other hand, the reduction of bone tissue is not related to an increased osteoclast activity because no changes were detected in the number of multinucleated Tartrate-Resistant Acid Phosphatase (TRAP) positive osteoclasts per mineralized surface ([N.Oc/MS per mm], AML-injected *vs* control implants: 69.52 ± 10.76 *vs* 62.83 ± 9.665) and in the osteoclast surface per mineralized surface ([Oc.S/MS]%, AML-transplanted *vs* control implants: 1.257 ± 0.26 *vs* 1.129 ± 0.319) (Fig.7D). Interestingly, mRNA expression levels of early-osteogenic markers SP7 and ALPL resulted up-regulated in AML-injected implants (ALPL, AML-injected *vs* control implants: 5.6 ± 2.308-fold; SP7, AML-injected *vs* control implants: 8.555 ± 1.882-fold), suggestive for a skew of BMSCs towards osteogenic commitment in presence of AML cells which however results ineffective for the full bone formation (Fig.7E). These data confirm that AML cells distort the osteogenic commitment of normal BMSCs *in vivo*, resulting in reduced bone formation in the absence of changes in osteoclastic activity.

Figure 7

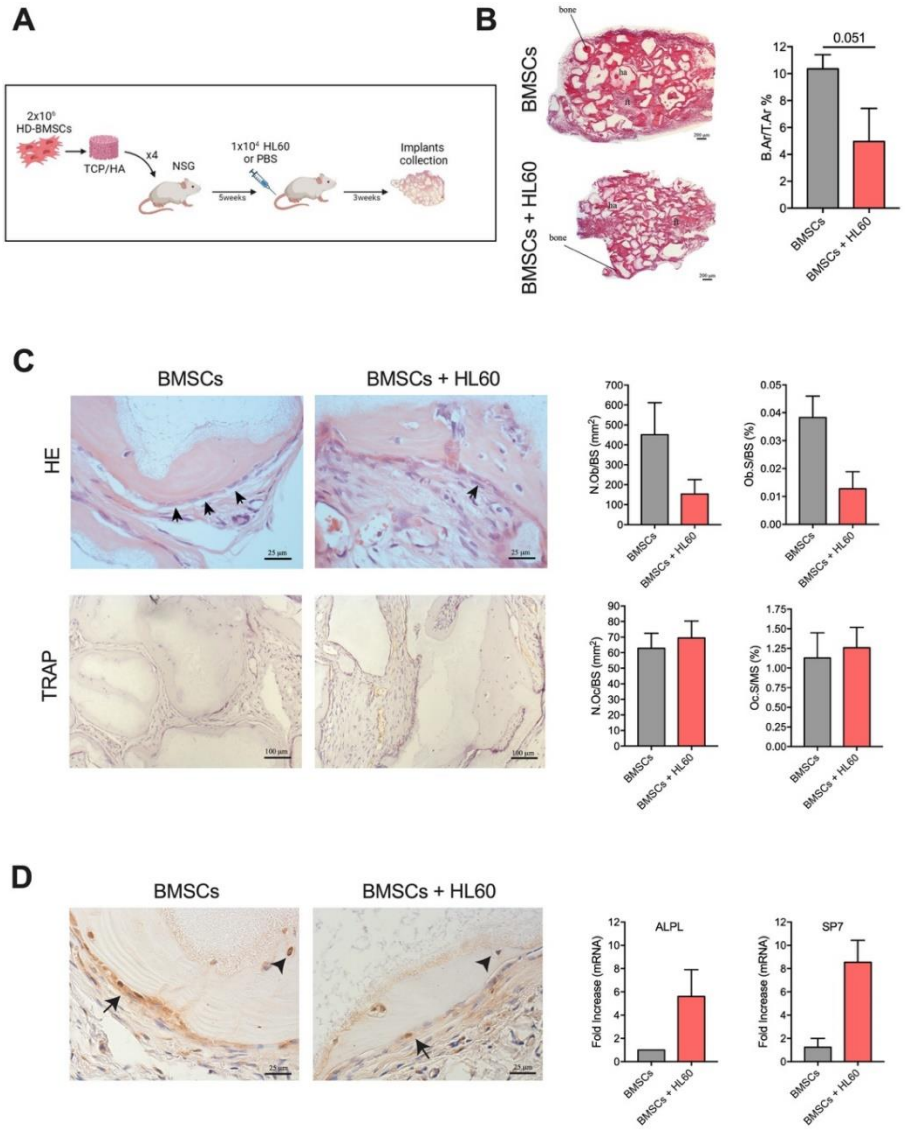

Figure 7. Impairment of *in vivo* osteogenic potential of normal BMSCs induced by AML cells. (A) Scheme of *in vivo* heterotopic subcutaneous osteogenesis transplantation assay. Normal BMSCs were loaded onto a HA/TCP carrier and implanted subcutaneously in NSG mice. 5 weeks after implant, mice were transplanted with HL-60 AML cells or PBS. The implants were harvested after 8 weeks *in vivo*. (B) Sirius red stained sections of AML-injected or control implants. Scale bars represent 200 µm. Histomorphometric analysis of bone

area/tissue area (B.Ar/T.Ar, n=6 control implants; n=4 AML-injected implants). Data are presented as mean ± SEM. (C) Hematoxylin and eosin (HE) stained sections showing osteoblasts covering bone surface in both controls and AML-injected transplants (arrows, scale bars=25 µm). Histomorphometric analysis of osteoblast number [N.Ob/BS], and osteoblast surface [ObS/BS]. Data are presented as mean ± SEM (n=2 control implants; n=4 AML-injected implants). Bottom panel shows representative images of sections stained for the osteoclastic marker TRAP from both AML-injected and control implants. Scale bars represent 100 µm. Quantification of Osteoclast number per mineralized surface (N.Oc/MS) and the mineralized surface (bone surface plus scaffold surface) lined by TRAP+ multinucleated osteoclasts (Oc.S/MS). Columns show the mean ± SEM (n=2 control implants; n=3 AML-transplanted implants). (D) Immunohistochemistry showing both osterix positive osteoblasts (arrows) and osteocytes (arrowhead). Quantification of mRNA level by qRT-PCR of early osteogenic genes Alkaline Phosphatase (ALPL) and Osterix (SP7) in AML-injected and control implants. Data are expressed as fold increase respect to control and presented as mean ± SEM (n=2 control implants; n=3 AML-injected implants). Statistical analyses were performed by unpaired *t*-test.

DISCUSSION

In this study, we showed that AML cells alter the osteogenic capacity of BMSCs and identified that Notch activation plays a key role in this process.

In the last decade, numerous evidence suggested that AML cells induce intrinsic alterations in BM niche components affecting their capacity to regulate and support normal hematopoiesis while favoring disease progression and therapy resistance[30–32]. In particular, several AML murine models have shown that bone tissue architecture was altered in leukemic mice[11,12,33]. Hanoun et al reported that murine AML BM contains a higher number of osterix-expressing osteogenic cells than healthy BM[11]. Moreover, we previously showed that BMSCs derived from AML patients are skewed toward an ineffective osteolineage differentiation with an accumulation of osteoprogenitors[13].

However, it is not clear how AML cells alter the osteogenic commitment of BMSCs. Deeply characterizing these alterations and associated pathways represents a critical step towards unraveling the mechanism underlying BM niche support of

disease progression and is paramount to finding new niche-targeted therapies for AML patients.

We assessed the osteogenic differentiation of normal BMSCs in the presence of AML cells combining *in vitro* and *in vivo* systems. Using a 2D co-culture system in which BMSCs and blasts were co-cultured in direct contact both in basal and osteogenic conditions, we found that AML cells, but not normal CD34+ cells, induced the upregulation of TNAP expression on BMSC surface. The direct interaction between AML cells and BMSCs is necessary to increase TNAP levels. TNAP is a marker of early osteoprogenitor cells[34] reported being overexpressed on osteoblast-primed AML patient-derived BMSCs compared with normal ones[21]. Furthermore, also osterix, another early-osteogenic gene, was transiently up-regulated on BMSCs exposed to AML cells, while late-osteogenic markers osteocalcin and osteopontin resulted decreased in BMSC induced to differentiate into mature osteoblasts in the presence of AML cells. These data can support an ineffective priming toward osteogenic differentiation induced by AML cells on BMSCs, with the generation of osteoblast precursors that cannot fully undergo successful complete maturation. Osteoblast maturation is indeed a multistep process in which BMSCs differentiate into osteoprogenitors, preosteoblasts, osteoblasts, and then osteocytes. Preliminary data obtained using a transplantation assay specific to assess the *in vivo* osteogenic potential of BMSCs[35] seem to confirm that HL-60 AML cells reduced the BMSC capacity to generate mature bone. Implants collected from human AML xenograft mice contain decreased bone tissue and reduced osteoblast number without changes in osteoclast formation. This observation provides further evidence that AML infiltration has a direct role in altering BMSC osteogenic commitment and agrees with findings obtained from AML murine models which display a significant inhibition of osteogenesis and a decreased number of osteoblasts associated with bone volume loss[33,36]. In addition, decreased osteogenic differentiation potential has been observed in adverse-risk AML patients[37]. Notably, Geyh and colleagues reported a reduced amount of osteocalcin, a small non-collagenous protein strictly correlated

with bone turnover, in BM and PB serum of AML patients, especially for those with adverse-risk disease[9].

We found that AML cells induce in BMSCs and their preosteoblast progeny a reduction in the expression of HSC-regulating genes, such as VCAM1, BMP4, and ANGPT-1, that could affect HSC retention and quiescence and exhaust the normal HSC pool[22–24]. Accordingly, in an AML patient-derived xenograft model, the progressive deregulation of these HSC-maintenance genes was associated with an increase in AML burden[38]. Furthermore, AML cells induce in BMSCs and derived preosteoblasts an increased expression of leukemogenesis-associated genes CCL2, CXCL8, and IL-6 involved in AML trafficking, proliferation, and chemoresistance[25–27]. These could represent potential molecular mechanisms by which BMSCs exposed to AML blasts could contribute to a massive proliferation of leukemic cells and failure of normal hematopoiesis.

Interestingly, we identified a possible role of Notch activation in AML-mediated alteration of BMSC osteogenic differentiation. Wnt/b-catenin and Notch pathways play an important role by regulating the crosstalk between AML cells and the stromal microenvironment and inducing AML progression[14–17,28]. BMSCs can enhance Notch signaling in AML cells via Jagged1 and rescue them from drug-induced apoptosis[14,15]. However, whether Notch ligands in AML cells can affect the osteogenic differentiation of BMSCs is unknown. We observed that Jagged1 and Dll1 were highly expressed in AML cell lines and primary AML blasts compared with normal BMNCs. In accordance, the same two ligands were reported elevated in the BM of patients with AML[39]. Activation of Notch signaling was observed in BMSCs after co-culture with AML cells, as demonstrated by the increased expression of Notch receptors and Notch target genes, such as Hes1 and Hey1. In addition, we found that the expression of TNAP was increased in BMSCs after Notch activation through immobilized recombinant Jagged1. Once γ-secretase inhibitor DAPT was added to the co-culture system, the TNAP up-regulation was prevented, supporting the hypothesis that AML cells can alter the osteogenic potential of BMSCs by activating Notch signaling. Moreover, after AML

exposure BMSCs up-regulated also the Notch ligand Jagged1, supporting an autocrine loop and making them potentially able to educate neighboring BMSCs toward a leukemia-permissive phenotype.

This implication of Notch as an important pathway to suppress functional osteoblast differentiation is not surprising as Notch activation has been shown to inhibit osteoblastic progenitor terminal differentiation through Hes1 repressing Runx2 transcriptional activity[40–42]. Similarly, in our co-culture system, we found that AML cells decreased RUNX2 expression and consequently the expression of mature osteoblast markers such as osteocalcin and osteopontin. Several evidence support that activation of Notch signaling in BMSCs induces early stages of osteogenic differentiation but prevents the formation of mature functional osteoblasts[19]. *In vitro* studies have shown that Notch activation in BMSCs or osteoprogenitors has a negative impact on osteogenic differentiation[43]. Engin et al observed that activation of Notch signaling *in vivo* induces the formation of bone structures with increased proliferation of immature osteoblast precursors[40]. Previous studies have already implicated the activation of Notch signaling in impaired osteogenic differentiation of BMSCs in other hematological malignancies, such as ALL[44,45], myelodysplastic syndromes[46], and multiple myeloma[47]. Although we showed that blocking Notch activation can partially recover the dysregulated osteogenesis, this does not exclude that other molecular mechanisms can contribute to AML-specific alteration of BMSC osteogenic commitment. Soluble factors[21], exosomes[12], and other contact-dependent pathways[16] have already been implicated in this process.

In summary, our results suggested that AML cells can generate a preosteoblast/osteoprogenitor-rich niche favorable for their growth by inducing ineffective osteogenic differentiation of BMSCs. Activation of the Notch signaling pathway not only induces AML cell proliferation and chemoresistance but can also be involved in the altered osteogenic differentiation of BMSCs exposed to AML cells. Therefore, Notch inhibitors might be an effective approach to target the interaction of AML cells and BMSCs and normalize osteogenesis in AML, adjusting the BM

niche. Further studies for better characterizing mechanisms used by AML cells to affect BMSC differentiation will increase our understanding of BM microenvironment alterations induced by blasts and provide the potential for new avenues of therapies.

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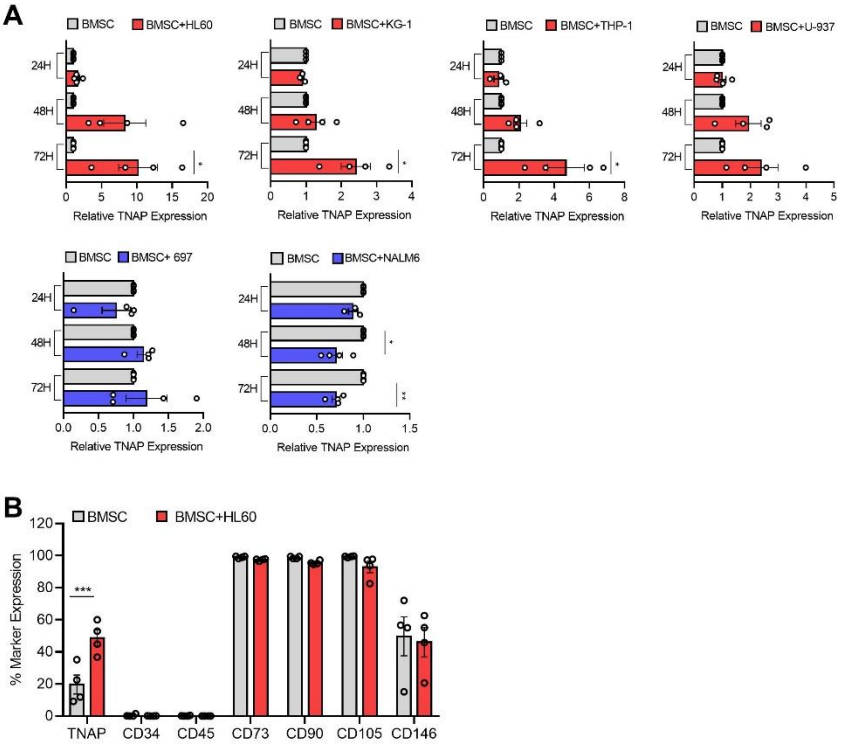
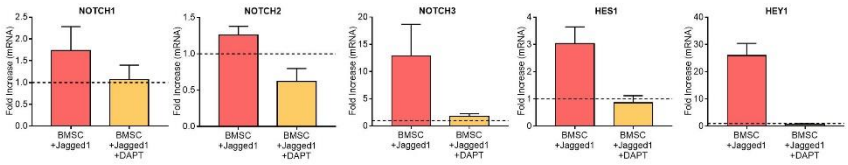
SUPPLEMENTARY FIGURES

Supplementary Figure 1



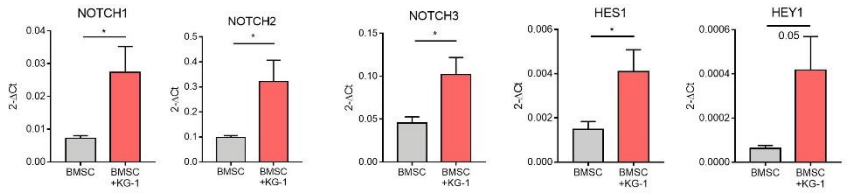
Supplementary Figure 1. AML cell lines induce a progressive TNAP over-expression on BMSCs without affecting other specific surface markers. (A) Relative TNAP surface expression on BMSCs was assessed by flow cytometry analysis after 24, 48, and 72 hours of co-culture with AML or ALL cell lines in basal condition (complete media). N=4 independent experiments using BMSCs from 6 different donors. Data are presented as individual values and the mean ± SEM. (B) Expression of BMSC surface markers (CD146, CD105, CD90, CD73, CD45, CD34) and TNAP on BMSCs assessed by flow cytometry after 72 hours of co-culture with HL-60 AML cell line. N=4 independent experiments using BMSCs from 4 different donors. Bars represent individual values and the mean ± SEM of % positive cells. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, by paired *t*-test.

Supplementary Figure 2



Supplementary Figure 2. The γ-secretase inhibitor DAPT abrogates the Notch signaling activation in BMSCs. qRT-PCR analysis of Notch signaling components performed on BMSCs after 72 hours of stimulation with immobilized recombinant Jagged1 in presence or absence of DAPT. n=3 independent experiments using BMSCs from 3 different donors. Data are expressed as fold increase respect to control and presented as mean ± SEM. *p < 0.05, by paired *t*-test

Supplementary Figure 3



Supplementary Figure 3. AML cell line KG-1 induces Notch signaling activation in BMSCs. qRT-PCR analysis of Notch signaling components performed on BMSCs in basal conditions (alone) or after 72 hours of co-culture with AML cell line KG-1. n=6 independent experiments using BMSCs from 6 different donors. Data are expressed as 2^{-DCt} and presented as mean ± SEM. *p < 0.05, by paired *t*-test

CHAPTER 5: DISCUSSION, CONCLUSION AND FUTURE PERSPECTIVES

The bone marrow (BM) niche is the physiological site in which hematopoietic stem cells (HSCs) reside and are finely regulated by stimuli from surrounding cells[1]. Among the hematopoietic regulators, bone marrow-derived mesenchymal stromal cells (BMSCs) are one of the most important, due to their multipotency allowing the generation of more differentiated cells, such as osteoblasts, chondrocytes, and adipocytes, which constitute the BM niche where HSCs live[2,3].

Acute myeloid leukemia (AML) is a hematologic neoplasm mainly induced by mutations in HSCs which lead to the proliferation of undifferentiated blasts in hematopoietic organs, like BM, lymph nodes, and spleen[4]. The growth of leukemic bulk leads to the hematopoietic failure. Despite substantial progresses in prognostic risk stratification[5] and personalized treatment with novel targeted drugs[6], AML patients still face a risk of chemoresistance and relapse that severely affects the outcome. Therefore, alternative therapeutic strategies are mandatory.

Once myeloid malignancies occur, the BM niche shows cellular and molecular alterations influencing disease manifestation, progression, and chemoresistance[7]. The role played by BMSCs and their progeny on the mechanisms influencing leukemia proliferation and survival during therapy is of potentially crucial importance for the development of new microenvironment-directed therapeutic strategies that may represent a valuable approach to complement existing treatments.

As detailed in our review submitted to Hemasphere journal, several evidence demonstrated that communication between AML cells and BM stromal cells is bi-directional. From one side, BM stromal cells regulate various biological processes in AML, from leukemogenesis[8–10] to chemotherapy resistance[11–13] and immune escape[14]. On the other one, leukemic cells can alter to their advantage the stromal compartment, inducing transcriptional and functional changes[15–17].

A growing research interest is focusing on the role of BMSC-derived osteolineage progeny in AML. Alterations in the osteoblastic compartment led to myelodysplastic syndrome and AML in mice[18–21]. In addition, osteoblasts can be remodeled by dysplastic cells to reinforce leukemia, with loss of mature osteoblasts and accumulation of osteoprogenitors[15,22,23].

There are several discrepancies among existing data on the properties of BMSCs isolated from patients with AML, particularly about their capacity to undergo osteogenesis[24–27].

In the first part of my PhD project, I studied the intrinsic alterations of AML patient-derived BMSCs (AML-BMSCs) resulting from their pre-existing interaction with leukemic cells, especially focusing on their osteogenic commitment.

In the first work, published in Haematologica journal[28], we compared BMSCs derived from AML pediatric patients at the time of the diagnosis with those from age-matched healthy donors. Despite AML-BMSCs appearing comparable to control in terms of morphology, colony-forming efficiency potential, proliferation, immunophenotype, and trilineage differentiation capacity *in vitro*, the use of two *in vivo* osteogenesis assays consented to highlight important peculiarities. These *in vivo* systems are more predictive than conventional *in vitro* differentiation assays of the intrinsic physiological potential of the cells[29]. The first assay used, based on the heterotopic subcutaneous transplantation of BMSCs loaded onto an osteoconductive carrier[3], revealed that AML-BMSCs maintained their *in vivo* bone differentiation capacity but generated a reduced amount of bone tissue. These results corroborate those of Frisch et al, which described a reduction in mineralized bone tissue formation in an immunocompetent murine model of AML[30]. Moreover, AML-BMSCs seem to present an altered osteogenic differentiation/maturation, as suggested by the phenotype of AML-BMSC-derived osteogenic cells found in implants, which express the early-stage marker osterix but not the mature osteoblast marker osteocalcin. Furthermore, osteocytes found in AML-derived implants are negative for the expression of Dentin matrix acid phosphoprotein 1 (DMP1), confirming the impairment of a complete osteogenic differentiation/maturation capacity. No

alterations were observed in terms of osteoclast number. Considering that perturbation of AML-BMSC osteogenic differentiation was evident also when they were removed from direct contact with blasts, a specific methylation signature affecting pathways involved in skeletal differentiation may be involved. Notably, Geyh et al. reported DNA methylation aberrancies in AML-BMSCs[24]. Specifically, among the most differentially methylated genes, two transcription factors TBX15 and PITX2 may be responsible for the impaired osteogenic differentiation of AML-BMSCs[31,32].

The second *in vivo* osteogenesis assay used was based on heterotopic subcutaneous transplantation of BMSCs growth as unmineralized pellets in a chondrogenic differentiation medium[33]. In this model, cartilage pellets were progressively substituted by marrow through a process ("endochondral myelogenesis") by which the ability of BMSCs to form a BM cavity and a functional stromal niche may be evaluated. We found that AML-BMSCs can develop a supportive hematopoietic stroma and reconstruct an *in vivo* BM-like microenvironment. However, AML-BMSC derived-ossicles contained a significantly increased fraction occupied by adipocytes. This observation is in line with the increased amount of adipocytes found in BM sections of AML patient with poor prognosis, described by Lu et al[34].

In both our models only the stromal components of the bone/BM organoid are human because they are derived from human BMSCs. The sinusoidal circulation and the hematopoiesis are of murine origin. Such a setting was suited for our need to investigate the intrinsic differentiation potential of AML-BMSCs rather than the interaction between the AML-derived BMSCs and AML/normal hematopoiesis. The evaluation of the impact of this peculiar AML-BMSC-derived osteogenic niche on normal/leukemic hematopoiesis support will be relevant. For this reason, we plan to realize a fully humanized model system by transplanting the ossicle-bearing mice with human $CD34^+$ cells or with human AML cell lines or primary blasts. The altered niche could promote the release of quiescent HSCs and favor the leukemia engraftment.

In the second part of my work, I focused on the effect of direct leukemia-stroma contact on the osteogenic potential of normal BMSCs. We used an *in vitro* 2D co-culture model to provide evidence supporting the concept that normal BMSCs, after contact with AML cells, altered their osteogenic commitment. BMSCs exposed to AML cells over-expressed TNAP, a marker of osteoprogenitor cells[35], and under osteogenic conditions do not increment the expression of late-osteogenic markers, such as osteocalcin and osteopontin. These data support the hypothesis that AML cells induce on BMSCs an ineffective priming toward osteogenic differentiation, with the generation of osteoblast precursors that cannot fully undergo successful complete maturation.

Preliminary data obtained using the *in vivo* osteogenesis assay, transplanting normal BMSCs loaded onto an osteoconductive carrier in AML xenograft mice, corroborated the *in vitro* results. Implants harvested from AML-injected mice presented a slightly reduced amount of bone tissue, with a decrease in the osteoblast count in the absence of alterations in the osteoclast number. Interestingly, mRNA expression levels of ALPL (the gene encoding for TNAP protein) were increased in AML-injected implants compared to control, suggesting a possible skew of BMSCs towards an incomplete osteogenic commitment. These data need to be further confirmed.

Our *in vitro* data supported that AML-mediated alteration of BMSCs osteogenic commitment is promoted by cell-contact. We found that Notch signaling can have a role in this perturbation. Activation of Notch signaling was observed in BMSCs after exposure to AML cells, as demonstrated by the increased expression of Notch receptors and their target genes. In addition, we found that the expression of TNAP was increased in BMSCs after Notch activation through immobilized recombinant Jagged1. Finally, by adding γ-secretase inhibitor DAPT to the co-culture system, the TNAP up-regulation was prevented supporting the hypothesis that AML cells can alter the osteogenic potential of BMSCs by activating Notch signaling.

Some evidence is already reported about the central role of Notch signaling in the crosstalk between AML cells and the BM

stroma microenvironment. In fact, Notch signaling has been involved in the BMSC-mediated support of AML cell survival[36] and resistance to chemotherapy[37]. On the other hand, modifications in the Notch signaling occurring in the BM stroma have been correlated to leukemogenesis[10,19]. To our knowledge, Notch signaling has not yet been implicated in the alteration of BMSC osteogenic differentiation induced by AML cells. A similar role is already been described in other hematological malignancies, such as acute lymphoblastic leukemia[38,39], myelodysplastic syndromes[40], and multiple myeloma[41]. This is not surprising, as Notch activation has been shown to inhibit osteoblastic progenitor terminal differentiation through Hes1 repressing Runx2 transcriptional activity[42–45].

Although we showed that blocking Notch activation can recover the dysregulated osteogenesis, we do not exclude that other different mechanisms carried out by AML cells may be responsible for perturbing osteoblast maturation, including the release of soluble factors[26], exosomes[23], and other signaling pathways[27]. Further *in vivo* studies of Notch signaling and the other mechanisms involved in altered BMSC differentiation into osteoblast in the context of AML are required to lead to the identification of rational therapeutic targets.

Considering that Notch signaling seem to play a double role in crosstalk between AML and stromal cells, promoting the AML cell survival mediated by the stroma[36,37] and negatively regulating the osteoblastic differentiation of stromal cells, its inhibition may represent a new strategy to target the interaction of AML cells and BMSCs and to normalize osteogenesis in AML patients. Notch pathway can be targeted at different levels of their cascade through several inhibitors, including blocking antibodies, decoy receptors, transcriptional inhibitors, and members of the family of γ-secretase inhibitors, some of them already used in clinical trials with different results in terms of outcome and toxicity[36]. Therefore, we will evaluate whether restoration of the normal activity of Notch pathway and in turn the BMSCs osteogenic commitment may synergize with standard chemotherapy agents and represent a good candidate for a dual targeting approach.

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1. Chiara Tomasoni, Alice Pievani, Benedetta Rambaldi, Andrea Biondi and Marta Serafini «Question of frame: The Role of the Niche in Myeloid Malignancies**».** Hemasphere. **Under revision.**
2. Chiara Tomasoni, Corinne Arsuffi, Alice Pievani, Samantha Donsante, Mara Riminucci, Andrea Biondi and Marta Serafini «AML alters Bone Marrow Mesenchyaml Stromal cell osteogenic commitment via Notch Signaling**». Manuscript in preparation**