Integrated OMICs unveil the bone-marrow microenvironment in human leukemia

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In brief
Passaro et al. provide a large omics repository of the BM microenvironment of immunodeficient mice. They then describe how primary human AML cells alter the molecular program of these niche components. Via integration of BM secretome and transcriptome data, they provide a repository of potential niche biomarkers.

Highlights
- Provide a large omics repository of the BM microenvironment of immunodeficient mice
- Demonstrate the transcriptomic overlap between different reporter niche markers
- Reveal particular pathologic crosstalk between AML and different niches
- Integrate multi-omics to predict niche biomarkers and therapeutic targets
Integrated OMICs unveil the bone-marrow microenvironment in human leukemia

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SUMMARY

The bone-marrow (BM) niche is the spatial environment composed by a network of multiple stromal components regulating adult hematopoiesis. We use multi-omics and computational tools to analyze multiple BM environmental compartments and decipher their mutual interactions in the context of acute myeloid leukemia (AML) xenografts. Under homeostatic conditions, we find a considerable overlap between niche populations identified using current markers. Our analysis defines eight functional clusters of genes informing on the cellular identity and function of the different subpopulations and pointing at specific stromal interrelations. We describe how these transcriptomic profiles change during human AML development and, by using a proximity-based molecular approach, we identify early disease onset deregulated genes in the mesenchymal compartment. Finally, we analyze the BM proteomic secretome in the presence of AML and integrate it with the transcriptome to predict signaling nodes involved in niche alteration in AML.

INTRODUCTION

Hematopoietic stem cells (HSCs) reside in a unique microenvironment referred to as the “niche,” which regulates the balance between self-renewal and differentiation of the hematopoietic stem compartment. The bone-marrow microenvironment is a complex multicellular tissue, whose non-hematopoietic component has been studied mostly in relation to its supportive function of hematopoietic lineages. Based on elegant in vivo depletion or expansion experiments and imaging-based approaches, a number of cell types have been suggested to contribute to the HSC niche. Studies on the bone-marrow (BM) stroma have defined individual populations in the stem cell niche regulating hematopoietic regeneration and capable of initiating leukemia (Crane and Kaeberlein, 2018; Yu and Scadden, 2016; Boulais and Frenette, 2015). To date, at least two functionally distinct perivascular niches that highly express cxcl12 and scf to dictate HSC cell fate have been identified: the arteriolar niches (Ding et al., 2012), composed mainly of arteriole-associated sympathetic nerve fibers, Nestinhigh Lepr+ cells are located (Morrison and Scadden, 2014; Boulais and Frenette, 2015). These distinct vascular beds have a specific spatial distribution relative to the bone (Sivaraj and Adams, 2016) and have been shown to differentially regulate hematopoiesis and osteogenesis (Itkin et al., 2016; Langen et al., 2017; Chen et al., 2019). Altersations of niche components has been described during the development of different blood malignancies (Kode et al., 2014; Goulard et al., 2018; Batsivari et al., 2020). Experimental models have highlighted several possibilities of crosstalk between malignant/premalignant cells and their niches as key contributors to disease initiation, progression, and resistance to therapy (Lané et al., 2009; Zhang et al., 2012; Miller et al., 2018). However, since the composition and molecular mechanisms used by the malignant niches are only partially understood, many unknowns remain.

High-throughput OMICS analyses on different interacting cellular components provide molecular insights into tissue-specific signaling networks with recent literature highlighting the
high complexity of the BM microenvironment transcriptome at single-cell resolution (Tikhonova et al., 2019; Baryawno et al., 2019; Baccin et al., 2020). The BM thus represents the optimal system to apply computational tools and decipher the mutual interactions between cellular components. Here, we used high-throughput RNA sequencing to perform genome-wide transcriptomic analysis of 7 sort-purified populations, by using our reporter mice for osteolineage cells (Osterix, Osx; Collagen, Collow and Colhigh) and different subsets of mesenchymal cells (Ng2; Nes high and Nes low) and CD31 markers for endothelial cells (Figure S1A).

First, we focused our attention on the molecular characterization of these stromal populations in homeostatic conditions (Figure 1A). Unbiased principal component analysis revealed tight clustering among stromal subsets (Figure 1B). Of all stromal subsets, CD31 and Col high were the most transcriptionally distinct. Likewise, mesenchymal components clustered together with a certain degree of overlap between populations. Nes high cells have been associated to peri-arteriolar cells (Kunisaki et al., 2013); however, their specific niche function has not been addressed. Interestingly, they show a specific transcriptional profile, only partially overlapping with Ng2 cells. A high degree of

Figure 1. BM niche populations identified with current markers
(A) Schematic figure of the experimental design depicting stromal components isolated from the BM of NSG mice carrying specific fluorescent reporters. FACS populations were analyzed by RNA-seq. CD31 n = 7; Nes high n = 4; Nes low n = 5; Ng2 n = 3; Osx n = 5; Collow n = 4; Colhigh n = 5.
(B) 3D PCA analysis showing similarities in expression profiles between different stromal components in healthy mice. Each dot represents an experimental replicate.
(C) Flow-cytometry analysis of CD45-Ter119+ BM cells derived from depicted mice showing overlap between markers associated to niche components.
(D) Correlation matrix showing similarities between different stromal components in healthy mice.
See also Figure S1.
overlap is observed between Osx, classically defined as osteo-
 progenitors, and Col low cells, while Nes low population sits in the middle of the plot, suggesting a certain degree of overlap with all the aforementioned populations (Figure 1B), which we confirmed by fluorescence-activated cell sorting (FACS) analysis (Figure 1C). Correlation matrix shows the degree of similarity between populations and unveils specific cellular overlaps (Figure 1D). For instance, Nes low (and to a lesser degree Nes high) cells partially overlap with CD31 cells, as previously reported (Figures 1C and 1D; Asada et al., 2017). Interestingly, a high degree of overlap is also observed between Ng2+ cells, classically considered as pericytes, and cells of the osteolineage (Figures 1C and 1D). Within the osteolineage, expression level of Col can be used to distinguish between different degrees of differentiation (Coutu et al., 2017).

Transcriptomic signature unravels specific features of BM niche cells in homeostasis

A specific cellular taxonomy of the BM non-hematopoietic niche has been recently defined via unbiased single-cell sequencing (Baryawno et al., 2019). We could classify our 7 BM niche populations based on these defined signatures (Figure S1B), confirming their conserved function in NSG mice. However, this classification only describes the transcriptome of niche cells at relatively low depth, a limitation associated with the single-cell sequencing technology.

To overcome this issue and unveil thorough molecular features of BM niche cells, we used their gene-expression profiles to define 8 functional clusters of transcripts responsible of the differences between populations (Figure 2A; Table S1). Analysis of biological processes and cellular functions allowed to define specific functional features of each cluster. By combining gene-expression values of each cluster and related processes, we can recapitulate the connections between niche cells in a process network map (Figure 2B).

Cluster 2 includes genes highly expressed in CD31 cells and specific for the endothelial function, such as endothelial commitment (pecam1, cdh5, kdr, emcn, cd34, flt4, ly6a, tie1, apln, pdgfb), vessel development (all1, dll4, notch1, notch4), regulation of angiogenesis (eng, flt1, ephb4, nck1), cell junction (gja4, gja5, gja6, cdh5, cdh6, jam2), and platelet aggregation (vwf, f2r, selp, cd36). Further classification of the genes in this cluster can be performed by comparative analysis with recently characterized subtypes of vascular endothelial cells (type L, H and E) (Langen et al., 2017) (Figure S2A). Cluster 1 identifies a heterogeneous group of genes highly expressed in osteolineage populations (Col high, Col low, Osx) as well as in Nes low and Ng2+ cells, and including genes involved in the deposition and interaction with the matrix (fap, CD44, mmp13, mmp16, tnc, thbs1-4, itgb1), connective tissue formation (col1a1, col1a2, dcn, sema3d), chondrocytic differentiation, cartilage formation (pth1r, met2c, runx2, fnb2), osteolineage (alp, sp7, runx2, spp1, smad9, dmp1, bglap1,2,3), and

Figure 2. Differential clusters between BM niche populations in homeostasis

(A) Cluster behaviors among niche components. Left: within-cluster averages of Z-scored expression profiles across stromal types from control samples (means ± SEM). Right: top enriched processes associated to each cluster.

(B) Process network map showing the relatedness of the different stromal types (size of each node proportional to the number of connections). See also Figures S1 and S2 and Tables S1 and S2.
bone formation (bmp1,3,5,7,8a). We noticed the lack of a proper mesenchymal stem cell (MSC) signature within this cluster, likely due to the low specificity of markers currently available to define such population. To dig into this question and try to isolate a cluster of genes specific to the MSC functions, we performed an analysis including gene expression of leptin-receptor+ (Lepr+) mesenchymal stromal cells from Tikhonova et al. (2019) and compared them to Neslow, NesEhigh, Ng2+ cells for their expression levels of genes in cluster 1. Interestingly, we identified two clusters of ~300 genes similarly expressed in the Lepr+ and Neslow cells and highly enriched in MSC signature genes (adipoq, lepr, cxxc12, cxxc14, gpx3, pdgfra, agt, grem1, igfbp5, foxc1, cdh2) (Worthley et al., 2015; Zhou et al., 2014; Zhao et al., 2019), which contains candidate markers to better define MSC population (Figure S2B; Table S2). Cluster 3 defines a small group of genes highly expressed in Neslow and Ng2 involved in skeletal functions, such as muscle system processes (acta1, myh1, myf1, tnn2, tnt3), skeletal development and cell-matrix interaction (acan, chad, col10a1, col27a1, col9a1, lect1), and cytoskeleton rearrangement (tubb4a, acta4, myh11). Cluster 5 includes genes highly expressed in NesEhigh, Ng2 and Neslow, involved in smooth muscle contraction (acta2, myh11, tm2) and regulation of blood pressure (acta2, acc2, atp1a2, notch3). Cluster 6 contains genes highly expressed in NesEhigh cells, involved in neuronal-related processes, such as nervous system development (ngfr, scn5a, egr1, agrt1a, trnsf12a), synaptic plasticity and transmission (cpeb1, egr1, syn, grin2a, pdlim4, prokr1, nos1), and neuromuscular junction (ank3, chna1, musk). Cluster 4 contains genes involved in immunoregulatory functions, such as myeloid cell differentiation and activation (mpo, lcn2, ltf, cybb, retifl), neutrophil chemotaxis (csar1, cxxc2, cxcr2, itgam, itgb2, nckap1l, syk), as well as myeloid-derived suppressor cells function (s100a8, s100a9, ngp). This cluster is particularly high in osteolineage and endothelial cells, pointing at a specialized function for these niche components in the BM innate immunity regulation. Cluster 7 includes genes highly expressed in NesEhigh and Ng2 cells mostly involved in mitochondrial function such as oxidative phosphorylation, ATP production and NADH metabolism (mt-atp8, mt-co1, mt-cytb, mt-nd1, mt-nd2, mt-nd4, mt-nd5, mt-nd6). Cluster 8 is particularly high in NesEhigh and CoEhigh cells and mainly contains genes involved in cellular anabolic processes, such as RNA processing and translation, ribosome assembly, and peptide metabolism (38 rpl and 31 rps genes, elf3h, elf1a1, uba, ubb, snrnp, naca) (Figures 2A and 2B). Overall, our clusters analysis allowed the identification of groups of genes/functions defining unique features as well as shared properties among niche populations.

**Human AML alters the homeostatic functional identity of niche cells**

We next analyzed how the presence of human AML engraftment impacts on the molecular signature of BM niche components. We used several donor BM patients representing different subgroups of the human AML disease spectrum (Table 1) as well as human AML cell lines and healthy human hematopoietic cells (CB) to engraft unconditioned immunodeficient mice, and isolated BM stromal cells as depicted in Figure 3A. t-distributed stochastic neighbor embedding (tSNE) analysis showed that AML does not impact on the global cellular identity of these niche components, since most of the replicates of each population reside in confined groups irrespective of belonging to a healthy or BM with AML (Figure S3A). We used our previously described clusters to unravel the AML impact on BM niche function. Some of the clusters remain stable upon AML engraftment, while others lose their expression trend (Figures 3B and S3B).

The overall signature of cluster 2 remains stable. However, we can identify changes in expression of specific subsets of genes, particularly the one associated with endothelial progenitor signature (Figure 3C). In fact, most of the upregulated transcripts correspond to genes highly expressed in type E cells, defined as progenitor/immature endothelial cells involved into matrix remodeling as well as angiogenesis and osteogenesis upon radiation injuries (Langen et al., 2017; Chen et al., 2019; Figure S3C). We also notice signs of endothelial-to-mesenchymal transition (EnMT; Figure S3D), a process already observed in myelofibrosis (Erba et al., 2017) and downregulation of genes involved in hemostasis (Figure S3E), suggesting a potential role of the endothelial component in severe complications of AML disease such as hemorrhages (Balmages et al., 2018).

Skeletal processes involved in bone and cartilage formation appear affected by AML, as shown by downregulation of specific genes in cluster 1 and 3 in Neslow and CoElow cells (Figure 3D). Interestingly, the same genes are instead upregulated in NesEhigh cells, pointing at a potential compensation mechanism (Figure S3D). In line with the EnMT phenotype, matrix and bone remodeling genes are also upregulated in CD31 cells (Figure S3F). The neurogenesis function seems downregulated in most of the mesenchymal cells (Neslow, NesEhigh, Ng2), while some specific genes are upregulated in CD31 cells (Figure S3G).

Another process highly affected by the presence of AML is the one associated with the smooth muscular function, as shown by downregulation of genes of cluster 5 in Neslow, NesEhigh, and Ng2 involved in muscle contraction and vascular tone regulation (Figure 3E).

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**Table 1. AML patient-derived samples**

| Sample ID | Karyotype | FAB | Age | Sex | NPM | FLT3 |
|-----------|-----------|-----|-----|-----|-----|------|
| AML1a     | Inv(3)(q21q26)/t(8;13)(p21) | M0  | 39  | M   | WT  | WT   |
| AML2b     | trisomy 13 | M1  | 67  | F   | Mut | n.a. |
| AML3ab    | normal    | M2  | 72  | F   | Mut | WT   |
| AML4a     | T(6;11) (Q27;Q23) | M5a | 36  | M   | WT  | WT   |
| AML5ab    | normal    | M4  | 24  | F   | WT  | ITD  |
| AML6b     | Del7q+8+11q | M4  | 61  | F   | WT  | ITD  |
| AML7a     | 1q        | M5a | 53  | M   | Mut | WT   |
| AML8b     | normal    | M1  | 61  | M   | Mut | ITD  |

Patient-derived AML samples used in the study and their genetic and clinical characteristics. n.a., not applicable.

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aSamples used for transcriptome experiments.

bSamples used for secreteme experiments.
On the same line, most of the genes of cluster 7 are downregulated in Ng2 and Nes\textsuperscript{high}\textsuperscript{p} cells (Figure 3F), pointing at a decreased metabolic function in muscular cells.

Finally, the BM innate immunity regulation function in cluster 4 is massively downmodulated in the presence of AML, both in the osteolineage and endothelial cells (Figure 3G).

Parallel to cluster-tailored analysis, we also performed an unbiased analysis of the global transcriptome deregulation of leukemic BM niche components. We used the top altered genes in each stromal type (Table S3) to reveal specific deregulation patterns estimated as hidden features from the data (Figure S4A). These deregulation patterns were either specific or shared across cell types, uncovering potential stromal relationships in the context of AML.

We build a global map highlighting the top deregulated processes and their behavior across stromal types (Figure S4B). Notably, we find shared patterns across osteolineage and endothelial cells in terms of downregulated processes, such as ion transport, cell adhesion, and immune regulation. Interestingly, while the process of vascular morphogenesis is similarly downregulated in both endothelial and osteolineage niche components, the pro-angiogenic response is specifically upregulated in the endothelial compartment and downregulated in the osteolineage, suggesting different processes driven by the two stroma cell types. Similarly, we evidence several common clusters of upregulated processes across the mesenchymal components, including a pro-proliferative response, cell-matrix interaction, connective tissue degradation, and specific signaling pathways such as Notch, NfkB, Esr1, and Wnt. Overall, our analysis shows multiple injuries to the homeostatic balance upon human AML engraftment in the BM niche specific for a single cell type or involving multiple components and potentially associated to AML pathologic phenotypes (Figure S4B).
Transcriptomic analysis at early time points unveils the progressive detriment of the BM mesenchymal niche in AML

Our data shed light on global molecular changes of the BM niche in the presence of fully established leukemia, in a disease setting mimicking the clinical scenario of patients at diagnosis. However, little is known about the dynamics of niche deregulation in the BM at early disease onset. To investigate whether any of the observed molecular changes could be detectable during the early onset of the disease, we used a recently developed system allowing identification and isolation of niche cells in close proximity with leukemia via transfer of a soluble fluorescent lipid-permeable mCherry (sLP-mCherry) marker (Ombrao et al., 2019, 2021), thus allowing molecular studies of the niche at early stage of disease. We focused our attention on the most abundant component of the niche, Nestin+ mesenchymal cells (Neslow), due to the challenging low number of retrieved niche cells at early time points. Upon engraftment with human leukemic cells expressing the sLP-mCerry, we isolated cherry+ Neslow (ch+) labeled early leukemic niche cells, and cherry− Neslow (ch−) cells present in the portion of the same BM, but not in contact with leukemic cells (Figures 4A and 4B). We compared the transcriptomic profile of ch+ Neslow cells from the early niche (1%–10% engraftment, “early”) and Neslow cells from heavily engrafted mice (70%–80% engraftment, “late”), a time point where all cells are in contact with leukemic cells. We observed that a big proportion of the AML-dependent molecular changes highlighted at a late time point were already present in the early niche. For instance, 40% of the genes deregulated in late Neslow cells were also significantly deregulated in early ch+ Neslow cells (Figures 4C, 4D, and S5), while only the 0.05% of them were altered in ch− Neslow cells (Figure 4D). This highlights the great potential of this approach to achieve specific physical isolation of the early niche in contact with AML cells. Our analysis allowed the identification of a large set of genes and pathways (Figures 4D, 4E, and S5) deregulated in the early crosstalk between mesenchymal cells and leukemia, which represent strong candidates as potential biomarkers for early AML diagnosis or targets for the rescue of the pathological phenotypes associated with the mesenchymal niche.

The BM secretome of patient-xenografts provides a reservoir of potential direct mediators of AML pathogenesis

Our transcriptomic data unravel molecular insights into the response of the BM niche to the presence of AML, but how much of this response derives from a direct crosstalk is unclear. To gain insight into changes in the BM milieu upon human AML engraftment, we performed a proteomic analysis of the BM secretome in AML xenografts (Figure 5A). Although we observed a certain degree of heterogeneity among AML samples, possibly linked to genetic diversity in our cohort (Figure 5B; Table 1), we identified a large common secretome signature within the AML cohort (Tables S4 and S5). We focused our attention on the top commonly deregulated proteins and processes and validated their transatlantic impact by comparing our dataset to recently published proteomics data retrieved from healthy donors and AML patients, as well as to a large cohort of patient-derived transcriptomic dataset (Baggar et al., 2016; Çelik et al., 2020). We observed a broad correspondence between xenograft and human patients’ data, with more than 30% overlap (Figure 5C; Table S4). Among the top deregulated pathways validated with human data, we found as expected AML associated signaling such as the RAF/MEK/ERK and PI3K/AKT cascades; interestingly, we observed upregulation of proteins involved in the RET signaling (Epiregulin, Gfra-1, Shp-2), recently highlighted as a mediator of normal HSC maintenance and expansion (Grey et al., 2020). We also found increased production of proteins involved in the interaction with the extracellular matrix (Osteonectin, Thrombospondin-1, Fibronectin, a1-Antitrypsin). The downmodulated pathways are largely related to the immune function, with decreased dendritic cell chemotaxis (Cxc15, Cxc9, Ccl5), immunoregulatory function and cytology (Granzyyme H, MiCB, Granulysin, Ilt-2, CD48). Interestingly, the clotting cascade and platelet activation pathways are also downregulated (Tffi, Dtk, KLKB1, Prekallikrein, a2-Antiplasmin, Coagulation Factor X; Figure 5D; Table S4).

Integration of niche transcriptome and BM secretome predicts molecular nodes involved in niche alteration in AML

To predict direct interactions responsible of the BM niche and AML crosstalk, we integrated the secretome and transcriptome data. Ligand-receptor analysis allowed the identification of upregulated and downregulated nodes of interaction between the BM milieu and niche components (Figure S6). The interactome showing ligand-receptor pairs involved in specific pathways is summarized in Figure 6A and can be explored at https://giovannidiana.github.io/integratedomics/. Overall, we noticed a peculiar dual distribution of signaling pathways between the osteo-endothelial niche and the mesenchymal components (Figure 6A). Among the others, we noticed an interesting combination of pro- and anti-angiogenic cues associated to multiple stromal types, suggesting distinct local regulation (Figure 6B). Interestingly, several signaling mediators of the complement and coagulation cascade as well as platelet activation and...
hemostasis are downregulated (Figure 6C), in line with clinical reports pointing at bleeding disorders and thrombocytopenia in AML patients (Del Principe et al., 2017; Balmages et al., 2018; Ahrari et al., 2019; Just Vinholt et al., 2019). On the immune side, increase in chemokines involved in monocyte chemotaxis is predicted to interact with the deregulated mesenchymal niche (Figure 6D), while reduced mediators associated to neutrophil function are connected to the osteo-endothelial niche (Figure 6E), the compartment mostly involved in the regulation of innate immunity under homeostasis according to our cluster analysis (Figure 2A, 3B, and 3G). In conclusion, our integrated sequencing data with the BM secretome of AML xenografts allowed the identification of specific signaling nodes as candidate mediators of stromal alteration in human AML.

**DISCUSSION**

The BM microenvironment has gathered increasing attention over the last years. Far from being a simple monolayer of supportive cells, the “hematopoietic niche” has revealed a high degree of complexity when studied in vivo with functional genetics (Crane et al., 2017; Wei and Frenette, 2018). Several markers have been proposed to label specific supportive niches within the tissue. Nevertheless, the high degree of overlap between these markers have only recently been evidenced by us (Figures 1 and S1) and other groups in the field (Coutu et al., 2017). The advent of high-throughput sequencing techniques now allows the molecular characterization of these cells with unprecedented precision (Tikhonova et al., 2019; Baryawno et al., 2019; Baccin et al., 2020). Our data covering the transcriptome of the bone-marrow niche at high sequencing depth contribute to fill these gaps by providing a large repository of potential markers to better define specific cell populations (Figures 2 and S2). Among the others, of high interest is the Nes high population, known for a long time but not studied in detail. Despite sharing some features with the other cell of mesenchymal origin, these cells appear strikingly different at the molecular level under homeostatic condition, highly expressing genes involved in RNA processing and translation (Figures 1 and 2). This peculiar feature could explain the ability of Nes high cells to rapidly adapt to a modified environment in the presence of leukemia and overcome the disrupted functions of other cell components (Figure 3), thus opening new perspective to deepen our knowledge on the nature and function of these cells. Moreover, we have also evidenced a remarkable association between the osteolineage and endothelial compartments, sharing specific functions in the regulation of tissue homeostasis and responding to leukemia insult as a highly connected unit (Figures 2, 3, and 6). A tight proximity between a

with healthy hematopoietic cells (CB) or human AML (AML; see Table 1 for details), and BM secretome was analyzed on a SOMAscan array. (B) 3D PCA showing similarities in protein expression profiles between samples. Each dot represents an experimental replicate. (C) Pie chart showing the number of deregulated proteins validated with comparison with depicted human datasets. (D) Top processes specifically upregulated (red) and downregulated (blue) in AML xenografts BM secretome compared to ctrl and validated on human data. See also Tables S4 and S5.
specific type of endothelial cells and the bone surface has been previously evidenced, with a prominent role in regulating angiogenesis, osteogenesis, and matrix remodeling (Kusumbe et al., 2014; Langen et al., 2017). Our analysis allowed the prediction of the local mutual interaction between these two compartments, further identifying a potential role in the regulation of innate immunity.

The presence of AML dramatically impacts on niche homeostatic function, as shown by our cluster-tailored analysis. Moreover, we highlighted that a pathologic crosstalk exists not only between AML and different niche cells but also within niche fractions. These intra-stroma interactions add another layer of complexity but also new potentials for drug interventions. The rebound of this extensive tissue deregulation on leukemia progression and normal hematopoiesis has only started to be addressed (Passaro et al., 2017; Duarte et al., 2018), and our data open novel paths for further studies of great relevance to the field. At our knowledge, no methods are available to date to isolate the living leukemic niche at single-cell resolution and at early time points of the disease. Laser-microdissection (Pacheco et al., 2018) or pipette aspiration (Silverstein et al., 2016) methods in bones have proved to have several limitations, notably poor resolution, age constraints, as well as the requirement for complex systems and technologies. The painting tool we have used (Figures 4 and S5) was designed to detect the early metastatic niche in lung cancer with wide-range flow cytometry techniques (Ombrato et al., 2019), and we have proved its applicability to isolate and study living hematopoietic niche cells at an early stage of leukemia dissemination in comparison to full-blown disease, producing data with great potential of unravel early mechanisms responsible for the progressive alteration of the leukemic niche.

The data presented in this work provided a large omics repository of the environment of immunodeficient mice. Despite the profound immunological defects of this model, it is widely used to produce impactful insights in the field of tumor xenografts, including the leukemic microenvironment (Abarrategi et al., 2018; Medyouf, 2017). Moreover, our results showing no major differences between our niche components and the functional clusters defined in immunocompetent mice in homeostasis (Tikhonova et al., 2019; Baryawno et al., 2019; Figure S1) as well as similarity between the deregulated proteomic signature in our xenotransplant model to the one reported in AML patients (Figure 5) endorse the validity of the xenograft model to study the non-hematopoietic BM microenvironment. Nevertheless, we recognize a certain number of limitations, notably the lack of the adaptive immune system. While the impact of AML on the immune environment has been recently addressed in patients (van Galen et al., 2019), very little is known about the non-hematopoietic niche at the molecular level in the human BM. It remains thus necessary to move these molecular studies a step forward in the translation process, by sequencing the human BM niche in its entirety and identifying specific markers to label functional environmental components in the regulation of normal and malignant hematopoiesis.

AML is a dreadful disease and in most cases is extremely difficult to treat in an efficient manner. There is a constant effort from the scientific community to try and identify novel therapeutic...
strategies tailored on specific subgroups, which offer promising results (Konopleva et al., 2016; Tomlinson et al., 2019; Perl, 2019; Roboz et al., 2020). We and others (Passaro et al., 2017; Duarte et al., 2018; Guarnerio et al., 2018; Battula et al., 2017; Tabe et al., 2017) have provided strong evidence that the microenvironment is an additional active part of the leukemic unit. Thus, our approach analyzing the bone-marrow environment in response to AML patients’ samples derived from different subgroups allows the identification of common pathologic hallmarks of the niche in AML disease, with a great potential to serve as a repository of biomarkers and therapeutic avenues. Further studies using similar approaches could follow to dive into differences between AML subgroups and draw subgroup-tailored deregulation maps in the microenvironment, which could serve as potential targets for personalized medicine.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109119.

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AUTHOR CONTRIBUTIONS

D.P. conceived the study, designed, conducted, and performed the experiments and the analysis and wrote the manuscript. M.G.A. and G.D. designed and performed the computational and statistical analysis and wrote the manuscript. P.C. performed the statistical analysis. L.A.M., A.B., C.B.E., A.A., and A.W. performed experiments. L.O., I.M., and J.G. provided materials and constructive comments. D.B. conceived the study, designed experiments, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## KEY RESOURCES TABLE

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| CD45 30-F11 1 in 400 Mouse | eBioscience | 45-0451-82 |
| TER119 Ter-119 1 in 400 Mouse | eBioscience | 12-5921-82 |
| CD31 390 1 in 400 Mouse | eBioscience | 25-0311-82 |
| EasySep Human CD34 Positive Selection kit | StemCell Technologies | 17856 |
| EasySep Human CD45 Positive Selection kit | StemCell Technologies | 100-0105 |
| EasySep Mouse CD45 Positive Selection kit | StemCell Technologies | 18945 |
| InVivoMAb anti-human CD3 | Euromedex/ Bioxcell | BE0001-2 |
| **Bacterial and virus strains** |        |            |
| sLP-mCherry pRRL lentiviral backbone | Ombrato et al., 2019, 2021 | N/A |
| **Biological Samples** |        |            |
| See Table 1         | N/A    | N/A        |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| DNase I             | Sigma-Aldrich | D4527 |
| Collagenase         | Sigma-Aldrich | C0130 |
| Dispase II          | Sigma-Aldrich | D4693 |
| Heparin             | Sigma-Aldrich | H3149 |
| **Critical commercial assays** |        |            |
| SomaScan® Platform  | Somalogic | [https://lifesciences.somalogic.com/technology](https://lifesciences.somalogic.com/technology) |
| Ovation® RNA-Seq System V2 kit (part No. 7102) | Nugen | M01206 v9 |
| RNeasy Mini kit     | QIAGEN | 74106 |
| **Deposited data**  |        |            |
| RNA-sequencing data | Geo Bank | GSE148626 |
| Secretome data      | N/A    | Table S5   |
| **Experimental models: Cell lines** |        |            |
| HL60                | ATCC   | Cell Service, The Francis Crick Institute |
| ML1                 | ATCC   | Cell Service, The Francis Crick Institute |
| **Experimental models: Organisms/strains** |        |            |
| NOD-SCID IL2Rgnull (NSG) | Jackson Laboratory | 005557 |
| NSG-NESTIN-EGFP     | The original strain is a kind gift from Dr G. Enikolopov. Backcrossed to NSG background at the BRF, The Francis Crick Institute (generation 8) | N/A |
| NSG-NG2-DSRED       | Original strain from Jackson Laboratory (008241). Backcrossed to NSG background at the BRF, The Francis Crick Institute (generation 8) | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
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Materials availability
This study did not generate new unique reagents.

Data and code availability
The accession number for the transcriptome sequencing data generated in this study are deposited on Geo bank (reference number: GSE148626). Protein array raw data are available in Table S5.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human primary samples
Umbilical Cord Blood (CB) samples were obtained from normal full-term deliveries after signed informed consent. AML samples used for xenografts were obtained after informed consent at St Bartholomew’s Hospital (London, UK). Details are listed in Table 1. The collection and use of all human samples were approved by the East London Research Ethical Committee (REC:06/Q0604/110) and in accordance with the Declaration of Helsinki. Mononuclear cells (MNCs) were isolated by centrifugation using Ficoll-Paque TM PLUS (GE Healthcare Life Sciences, Buckinghamshire, UK). For CB samples, the cells were processed within 24 hours following collection. Cells were enriched for CD34+, using an EasySep Human CD34 Positive Selection kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer’s instructions, with a purity of 85 to 99%. T cells were depleted from all AML samples using the anti-CD3 mAb OKT-3 (West Lebanon, USA).
Mouse models

All animal experiments were performed under the project license (PPL 70/8904) approved by the Home Office of UK and in accordance to The Francis Crick institute animal ethics committee guidelines. Nestin-GFP (Mignone et al., 2004) mice were a kind gift from Dr G. Enikolopov. Col2.3-CFP (Visnjic et al., 2001) mice were a kind gift from Dr. DW Rowe. Osx-cre-Gfp (Rodda and McMahon, 2006) and Ng2-Dsred (Zhu et al., 2008) strains were purchased from Jackson laboratory. NOD-SCID IL2Rnull (NSG) strains were also obtained from Jackson Laboratory, Bar Harbor, Maine, USA and were bred at The Francis Crick Institute Biological Resources Facility in individually vented cages (IVCs) under Specific Pathogen Free (SPF) conditions. All reporter strains were backcrossed into the NSG background (generation 8 or more) at The Francis Crick Institute.

Cell lines

HL60 and ML1 cells were grown in RPMI1640. All cell lines were tested for mycoplasma prior to commencing experiments. HL60 came originally from ATCC (distributor LGC standards, UK), ML1 from European Collection of Authenticated Cell Cultures (ECACC) and were grown by our cell service at the Institute. Before using these lines, they were authenticated using the Short Tandem Repeat (STR) profiling. All media were supplemented with 10% FBS and 1x Penicillin-Streptomycin and all reagents were from Gibco®-Life Technologies (Paisley, UK). The sLP–mCherry sequence was produced and cloned into a pRRL lentiviral backbone as previously described (Ombrato et al., Nature 2019). Briefly, a soluble peptide (SP) and a modified TAT peptide were cloned upstream of the mCherry cDNA, under the control of a mouse PGK promoter. HL60 and ML1 cells were stably infected with sLP–mCherry lentiviral particles (MOI 10) and flow sorted 48 hours later to select the positive cells.

METHOD DETAILS

AML and HSPCs transplantation assay in vivo

For xenografts assays, human AML cell lines (HL60, ML1; 2x10⁶), Okt3-treated AML patient-derived samples (2-8x10⁶) and healthy HSPC (1x10⁶) were transplanted into 8 to 12 week old unconditioned mice by i.v. injection. Each experimental cohort contained an equal number of male and female mice. BM engraftment was assessed by BM aspirate. Mice were euthanized when human engraftment was > 50% (or 1%–10% for early time point experiments), with an age of 13 to 20 weeks.

Bone marrow cell isolation

At the end of each experiment, animals were euthanized by cervical dislocation and the 6 rear bones collected in cold PBS. To retrieve BM niche cells, bones were dissected in small pieces of 2-3 mm diameter and incubated 20 min at 37 degrees in a HBSS digestion solution containing 2% FBS, DNase I (10 μg/ml), Collagenase (1 mg/ml), Dispase II (5 mg/ml) and Heparin (20 U/ml), all from Sigma-Aldrich, Gillingham, Dorset, UK. Bone pieces were next crushed with mortar and pestle in the same digestion solution and incubated at 37 degrees for 20 min. Cell suspension was then homogenized with a micropipette and filtered with a 100 μm strainer. To retrieve BM extracellular proteins, the BM homogenized suspension was flushed into 100 ul PBS and spin at 80 degrees until analysis.

Flow cytometry analysis and cell sorting

Flow cytometry analysis was performed using a Fortessa flow cytometer (BD Biosciences, Oxford, UK). Dead cells and debris were excluded from the analysis using 4,6-diamidino-2-phenylindole (DAPI) or SYTOX Green (ThermoFisher) staining. Cell sorting was performed using a FACSAria SORP (BD Biosciences, Oxford, UK). Data were analyzed by FACSDiva (BD) or FlowJo (version 9.9) software. To sort BM niche cells, human and murine hematopoietic cells were excluded using an EasySep Human CD45 Positive Selection kit and a murine CD45 positive selection kit (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer’s instructions, with a purity of 85 to 99%. Remaining cells were stained with hCD45 (2B11), mCD45 (30-F11), Ter119 (Ter-119) and mCherry cDNA, under the control of a mouse PGK promoter. HL60 and ML1 cells were stably infected with sLP–mCherry lentiviral particles (MOI 10) and flow sorted 48 hours later to select the positive cells.

Sample preparation for RNA-sequencing

RNA was extracted using RNAeasy Mini kit (QIAGEN) following manufacturer instructions. The quality and concentration of total RNA were determined on Agilent 2100 Bioanalyzer using the Eukaryote Total RNA Pico Assay. Most of the total RNA has average RIN number 5-8, with a concentration at least 4 pg/µl. Some of the samples were concentrated on a speedvac without heat to obtain a final volume of 5 µL. 3.5 ng of Total RNA in 5 µL volume was used to generate cDNA synthesis with Nugen Ovation® RNA-Seq System V2 kit (part No. 7102). The resulting SPIA-cDNA were normalized to 100 ng in 15 µL based on Qubit DNA HS assay. Fragmentation was done using 8 microTUBE-15 AFA Beads Strip V2 (PN 520159) on Covaris E-series at setting 20%DF, 18W, 200 burst, 20C temp, 120 s treatment time and no Intensifier. Only 10-30 ng in 10 µL of fragmented cDNA was used starting from the End Repair step of the Ovation® Ultralow Library Systems V2 (part No. 0344NB) protocol, with 10 cycles PCR. The Rnaseq libraries were quality checked on Qubit DNA HS assay, Bioanalyzer and Illumina Ecoreal QPCR followed by Illumina PE51 sequencing on Hiseq 2500 V3 reagents.
Samples preparation for secretome
Supernatants from processed bones were spin down to remove precipitates and cellular debris. Protein concentration was quantified using Bradford assay and quality assessed via gel electrophoresis. 20 μg of proteins was used to perform a broad-scale proteomic analysis (Somalogic, Boulder, CO, USA) using their aptamer-based technology to quantitatively evaluate proteins present in the bone marrow aspirate between control mice and mice engrafted with AML samples. The SomaScan® assay quantitatively transforms the proteins present in a biological sample into a specific SOMAmer-based DNA signal. A SOMAmer-protein binding step is followed by a series of partitioning and wash steps that converts relative protein concentrations into measurable nucleic acid signals that are quantified using DNA detection technology, which for the SOMAscan assay with over 1,300 SOMAmer reagents is by hybridization to custom DNA microarrays. Assay details are provided in Gold et al. (2010).

RNA-sequencing analysis methods
FastQ files were generated using CASAVA BCL to FastQ (version 2.16). Sequencing yield was typically ~25 million strand-specific paired-end reads. The RSEM package (version 1.2.29; Li and Dewey, 2011) in conjunction with the STAR alignment algorithm (version 2.5.1b; Dobin et al., 2013) was used for the mapping and subsequent gene-level counting of the sequenced reads with respect to mm10 Ensembl genes downloaded from the UCSC Table Browser 15 on 14th April 2016 (Karolchik et al., 2004). The “-forward-prob” parameter was set to “0” and all other parameters were kept as default. Normalization was performed with DESeq2 package (median of ratios method) (version 1.22.2; Love et al., 2014) with subsequent regularization using log_{10} transformation. Differential expression analysis was then performed with the DESeq2 package under the R programming environment (version 3.2.3) (R Development Core Team, 2015). Genes with FDR < 0.1 were judged to be differentially expressed. Genes from each given pairwise comparison were ranked using the Wald statistic. To analyze the transcriptome of stromal components in human xenografts, dimensionality of gene expression from AML, CB and ctrl samples was reduced with the t-distributed stochastic neighbor embedding (tSNE) technique for 2D visualization using the plot.t_sne function from the bioplots package on R. A comparison analysis was then performed between AML and ctrl samples in order to obtain differentially expressed genes. This analysis was performed using the contrast function within DESeq2 package. Linear correlation coefficients were calculated for control (ctrl) samples and a sample distance heatmap was then obtained using the heatmap package under R environment. Dimensionality reduction analysis was performed using gene expression data from ctrl samples. Principal component analysis (PCA) was made by using the expression matrix of normalized and regularized genes using the prcomp function on R. The results were visualized on a 3D map using the plot3d function from the rgl package on R. Multiple comparison of stromal populations in control samples was performed within the DESeq2 computing environment. We performed pairwise comparisons of all stromal cell types generating fold changes of each gene in each comparison of stromal types. Significant genes included in cluster analysis were selected by requiring at least one significant fold change in the pairwise stromal comparison (adjusted p value lower than 0.05). Clustering of selected genes was performed by applying a density-based algorithm (Rodriguez and Laio, 2014) where peaks in the distribution of z-scored expression across stromal types are associated to cluster centers. The pipeline adopted for this analysis consists of (1) fast search of density peaks in the distribution of normalized counts, (2) assignment of genes to clusters (density peaks) and (3) exclusion of noisy genes located in regions of low density. Cluster compactness was quantified as the average Pearson’s correlation between the mean cluster profile and the z-scored profiles of genes within the cluster. To obtain insights on how different cell types mediate interactions among pathways we applied a force-based visualization technique. First, we built a bipartite network with two node types for stromal cells and relevant molecular processes and edges weighted by the interactions described above. Second, we run a standard under-damped dynamic with elastic attraction and electric repulsion among nodes to find their equilibrium configuration. In order to characterize ctrl cluster 2 into sub-clusters of genes enriched in specific BM endothelial functions a database from Langen et al. (2017) was downloaded and compared against genes present in cluster 2. This dataset divides endothelial cells into three main categories, sinusoidal endothelium cells (type L), specialized capillary subtype (type H), and specialized endothelial cell subtype (type E). A heatmap was obtained using the heatmap package on R with the option ward.D2 as a clustering method on row scaled data. To characterize genes present in ctrl cluster 1 specific to MSC functions, we performed a meta-analysis in which gene expression of leptin-receptor-positive (Lepr+) mesenchymal stromal cells from Tikhonova et al. (2019) were joined to the gene expression of Neslow, Neshigh, and Ng2 samples from our study. All datasets were joined in a matrix and normalized together using DESeq2. Normalization and regularization were done as described before obtaining gene expression data for the different cell types. Comparisons were done for those genes present in cluster 1 and a heatmap was performed using the heatmap package on R with the option ward.D2 as a clustering method on row scaled data. For the global transcriptome analysis, significantly deregulated genes sharing similar deregulation patterns between control and leukemic samples were classified into groups by applying a model-based Bayesian framework. Deregulation patterns were represented by vectors of length equal to the number of stromal types, with elements taking discrete values: 0 (non-significant regulation), 1 (upregulation) and −1 (downregulation). We adapted the generative model introduced in Diana et al. (2019) to our data to detect deregulation patterns.

Secretome analysis
Data was analyzed using the Bioconductor version 2.13 (http://www.bioconductor.org) running on R version 3.0.2 (available from www.R-project.org). Protein intensity values were quantile normalized and log2 transformed. Differences in protein expression between groups was assessed using an empirical Bayes moderated t-statistics test from the “limma” package. P values were adjusted
for multiple testing correction using the Benjamini-Hocberg method. PCA was made by using the expression matrix of normalized and regularized genes using the prcomp function on R. The results were visualized on a 3D map using the plot3d function from the rgl package on R. Ligand-receptor pairs database was downloaded from Ramilowski et al. (2015) and compared against protein expression of the secretome from somascan arrays and gene expression from the statistically significant comparisons of AML versus ctrl RNaseq. Two lists of pairs for each cell type were obtained with the secretome as ligand and cell gene expression as receptor. To generate the dynamic figure, we used ligand-receptor pairs which were found coherently up or down-regulated in any of the stromal types. We constructed a bipartite graph representing cell-specific ligand-receptor connectivity and used force-driven dynamics on the network nodes to visualize the structure. We combined these results within a github (https://github.com/). The graphic map of the ligand-receptor interactome was sketch draw by adapting the thickness of the connection lines to the number of interactions between each cell type and the secretome, and between cell types.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Information on number of replicates used in each experiment can be found in the figure legends. Details on OMICs analysis method and statistics can be found in Method details section. Based on sample size and intra-group variability in transcriptomic and proteomic data, significance was defined by an adjusted p value < 0.1.
Supplemental information

Integrated OMICs unveil the bone-marrow microenvironment in human leukemia

Diana Passaro, Manuel Garcia-Albornoz, Giovanni Diana, Probir Chakravarty, Linda Ariza-McNaughton, Antoniana Batsivari, Clara Borràs-Eroles, Ander Abarrategi, Alexander Waclawiczek, Luigi Ombrato, Ilaria Malanchi, John Gribben, and Dominique Bonnet
Figure S1. BM niche populations in homeostasis. (A) FACS gating strategy of BM niche cellular components. (B) Baryawno et al. clusters behaviors amongst niche components. Normalized z-scored across stromal types from control samples (means ± sem) is shown. Related to Figure 1 and 2.
Figure S2. Differential clusters between BM niche populations. (A) Heatmap showing expression levels of genes in cluster 2 in different BM endothelial subpopulations. (B) Heatmap showing expression levels of genes in cluster 1 in different mesenchymal stromal population. Sub-clusters enriched for MSC signature genes are highlighted (see table S2 for details). Related to Figure 2.
Figure S3. Human AML impact on BM niche homeostatic balance. (A) tSNE map of different stromal components in ctrl, healthy and AML xenografts. (B) Cluster compactness in ctrl (black) vs AML (red). (C) Heatmap showing expression levels of endothelial genes of cluster 2 unregulated in the presence of AML in different BM endothelial subpopulations. (D-G) Deregulation (mean log2FC) of genes in depicted clusters in the presence of AML (* means FDR < 0.1). Related to Figure 3.
Figure S4. Human AML impact on BM niche. (A) Heatmap showing the log2FC of the top deregulated genes in each stromal cell types, clustered based on machine learning pattern aggregation algorithm (see methods for details). (B) Process map based on mean FC of each pattern. Related to Figure 3.
Figure S5. Progressive detriment of the BM mesenchymal niche in AML at early time points. (A, B) Deregulation (Mean log2FC) of genes in depicted clusters in the presence of AML in Late and Early stage of leukemic engraftment in mesenchymal niche (Nes\textsuperscript{low} cells) (* means FDR < 0.1 and are depicted only for Early). Related to Figure 4.
**Figure S6. Integration of niche transcriptome and BM secretome.** Sketch graphs of upregulated (red) and downregulated (blue) predicted ligand-receptor interactions between BM secretome and niche transcriptome. Related to Figure 6.