

Microarrayed human bone marrow organoids for modeling blood stem cell dynamics

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ABSTRACT

In many leukemia patients, a poor prognosis is attributed either to the development of chemotherapy resistance by leukemic stem cells (LSCs) or to the inefficient engraftment of transplanted hematopoietic stem/progenitor cells (HSPCs) into the bone marrow (BM). Here, we build a 3D *in vitro* model system of bone marrow organoids (BMOs) that recapitulate several structural and cellular components of native BM. These organoids are formed in a high-throughput manner from the aggregation of endothelial and mesenchymal cells within hydrogel microwells. Accordingly, the mesenchymal compartment shows partial maintenance of its self-renewal and multilineage potential, while endothelial cells self-organize into an interconnected vessel-like network. Intriguingly, such an endothelial compartment enhances the recruitment of HSPCs in a chemokine ligand/receptor-dependent manner, reminiscent of HSPC homing behavior *in vivo*. Additionally, we also model LSC migration and nesting in BMOs, thus highlighting the potential of this system as a well accessible and scalable preclinical model for candidate drug screening and patient-specific assays.

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INTRODUCTION

Bone marrow (BM) governs hematopoietic stem/progenitor cell (HSPC) homeostasis and differentiation through its complex microenvironment,¹ which comprises two distinct HSPC niches: one in the microvessel-enriched trabecular region and another at the cortical regions of the bones.² Within these niches, HSPC behavior is tightly regulated by a multitude of cellular players, including mesenchymal stem/progenitor cells (MSCs), vascular endothelial cells (ECs), osteoblasts, sympathetic nerve fibers, macrophages, megakaryocytes, adipocytes, and non-myelinating Schwann cells.^{3,4} However, despite extensive control mechanisms, a variety of hematological and immunological diseases still occur, including acute myeloid leukemia (AML), with its defining characteristics attributed to the rapid accumulation of abnormal immature progenitor cells.^{5,6} Classically, the treatment of AML consists of chemotherapy followed by transplantation of healthy HSPCs, yet this often fails to

substantially improve patient prognosis.⁷ Relapses are mainly attributed either to the inability of the treatments to eliminate all leukemic blasts or the failure of injected donor HSPCs to engraft into the patient's BM; both failures are associated with a malfunctioning BM niche microenvironment. Thus, modeling human BM with its intricate stem cell–niche interactions is pivotal for the development of novel therapeutic strategies.

Classic *in vivo* models (e.g., xenograft mice) used to assess the homing capacity, engraftment, and reconstitution potential of donor HSPCs have significantly advanced the general understanding of HSPC behavior,⁸ though they remain tightly linked to ethical and technical constraints.⁹ Bioengineered cell culture systems have been proposed as potential alternatives for the manipulation and observation of HSPC behavior such as self-renewal and differentiation in niche-mimicking microenvironments.^{10–12} However, these *in vitro* systems are based on two-dimensional (2D) culture settings and, as such, poorly recapitulate

the complex and multicellular three-dimensional (3D) environment found in the native BM.

Stem cell-derived self-organizing structures, so-called organoids, have recently emerged as a revolutionary tool for basic and translational research.^{13,14} These multicellular *in vitro* structures recapitulate the cellular spatiotemporal organization that occurs during development, tissue homeostasis, and regenerative processes *in vivo*.¹⁵ However, despite extraordinary activity in the organoid field over the past decade, very few *in vitro* organoid model systems for BM have been described. The closest approximations are likely those developed through bioengineering techniques, such as “ossicle” scaffolds,^{16,17} which are based on the *ex vivo* formation of a human stromal cell layer followed by a subsequent ectopic transplantation. Such engineered ossicles show comparable morphological, phenotypic, and functional features to native bone organs, facilitating the probing of “humanized” microenvironments for human HSPCs *in vivo*.^{16,17} However, the dependency of this system on xenotransplantation remains a major drawback for standardization. A fully *in vitro* setup has also been established using a perfusion bioreactor system to form an engineered human osteoblastic niche.¹⁸ Even more relevantly, the formation of scaffold-free mesenchymal spheres, called mesenspheres, has been shown to preserve the multilineage potential of HSPCs by secreting HSPC-supportive cytokines.¹⁹ Despite mimicking the phenotypic and structural characteristics of the BM mesenchymal compartment, both of these systems still fail to recapitulate the multicellular context of the native BM. Two recent studies integrated such multicellular dimensions into “bone marrow-on-chip” platforms, built by a microfluidic channel containing stromal cells that is positioned parallel to a channel lined with endothelial cells.^{20,21} Partial maintenance of HSPCs and their multilineage differentiation has been observed in these systems, yet their wider applicability may be limited by the static and indirect interaction of the two cell types as well as the low scalability of the designs.^{20,21}

To close this technology gap, we have developed a novel approach for generating BM organoids (BMOs) with *in vivo*-like functional characteristics. Here, we see the self-organization of a pseudo-vascularized network within a mesenchymal compartment via the aggregation of the two major niche cell types, MSCs and ECs. Intriguingly, BMOs containing such an endothelial compartment displayed an enhanced recruitment of HSPCs, which then resided in close proximity to the endothelial network. Furthermore, this migratory behavior seems to be partially regulated in a chemokine ligand/receptor-dependent manner, similar to the *in vivo* process. We also provide proof-of-concept for the deployment of this system for disease modeling, particularly in AML. We, thus, believe that this organoid system holds great promise as a preclinical model for pharmacokinetic assays and toxicity studies of novel drugs in a BM niche-mimicking 3D environment.

RESULTS

Formation of bone marrow organoids in a scalable manner

Guided by the natural composition of BM, we selected two major cellular niche components, namely, MSCs and ECs, for the generation of BMOs. We used a commercially available microwell platform consisting of a high-density array of round-bottom poly(ethylene glycol) (PEG) hydrogel microwells (Gri3D[®]) to generate BMOs in a high-throughput manner [Fig. 1(a)].²² Once sedimented at the bottom of the microwells, the two cell types formed a compact

mesenchymal spheroid featuring a morphologically distinct endothelial compartment [Fig. 1(a)]. Considering the essential role of MSCs and ECs in the recruitment and maintenance of HSPCs,^{23,24} we hypothesized that these cells could trigger the homing and engraftment of HSPCs and leukemic blasts in BMOs, thus mimicking organ-level physiological processes [Fig. 1(a)].

To define the optimal 3D culture condition for the formation of BMOs, we first tested the aggregation capacity of human MSCs and human umbilical vein endothelial cells (HUVECs) in two different ratios, namely, 50/50 and 75/25, respectively, compared to a 100% MSCs control condition (100/0)¹⁹ [Fig. 1(b)]. Bright-field imaging demonstrated an efficient and robust generation of almost 3000 BMOs per Gri3D 24-well plate (121 BMOs/well) [Fig. 1(c); supplementary material Fig. 1(a)]. After seven days in culture, all tissues stained positively for the mesenchymal marker endoglin (ENG) and the endothelial marker CD31 for dual-cell type conditions (50/50; 75/25) [Fig. 1(d), supplementary material Video 1]; HUVECs seeded alone in the microwells neither aggregated nor survived (data not shown). The largest aggregate growth was detected in the 100/0 control condition followed by the 75/25 condition, and the smallest growth was detected for the 50/50 condition [Fig. 1(e)]. Increasing the percentage of ECs resulted in smaller, yet more reproducible, aggregate sizes, which was also confirmed by quantitative volumetric analyses [Fig. 1(f)]. These data demonstrate that our high-throughput culture method offers a robust and efficient formation of large quantities of BMOs composed of MSCs and ECs.

Self-renewing and differentiated MSCs in bone marrow organoids

We next characterized the composition and identity of the mesenchymal compartment within BMOs by assessing the presence of key phenotypic markers. Immunofluorescence analysis revealed comparable expression levels among all conditions for nestin (NES) and transgelin (TAGLN), two mesenchymal factors involved in hematopoietic stem cell (HSC) recruitment and blood vessel formation *in vivo*.^{19,25} [Figs. 2(a) and 2(b); supplementary material Fig. 2(a)]. Interestingly, we found a higher expression of the self-renewal mesenchymal marker ENG in the dual-cell type BMOs (75/25; 50/50) compared to the control condition (100/0) [Fig. 2(c); supplementary material Fig. 2(a)]. To quantitatively assess the portion of multipotent, self-renewing MSCs characterized by the CD71⁻/CD31⁻/CD45⁻/ENG⁺/CD146⁺ immunophenotype,^{19,24} we analyzed digested BMO cells by flow cytometry [supplementary material Figs. 2(b) and 2(c)]. Within the CD71⁻/CD31⁻/CD45⁻ mesenchymal cells, we observed a higher mean fluorescent intensity (MFI) for ENG in the dual-cell type BMOs, supporting our immunofluorescence observations, while the CD146 levels remained constant [supplementary material Fig. 2(d)]. In terms of cell numbers, we detected an increase in up to 30%–50% of the ENG⁺/CD146⁺ double-positive (self-renewing) phenotype in both dual-cell type conditions compared to the mesenchymal control condition [Fig. 2(d); supplementary material Fig. 2(e)]. These findings show that the phenotypic self-renewing MSCs are more abundant in the dual-cell type bone marrow organoids, possibly through a supportive interaction between the ECs and MSCs, compared to HSPC recruitment or blood vessel formation.

Using histology, we next asked if MSC-derived differentiated cell types appeared in BMOs. Hematoxylin and eosin (HE) staining showed that the majority of the cells grow at the periphery of the structures, while the center appears as a dense eosinophilic core [light pink,

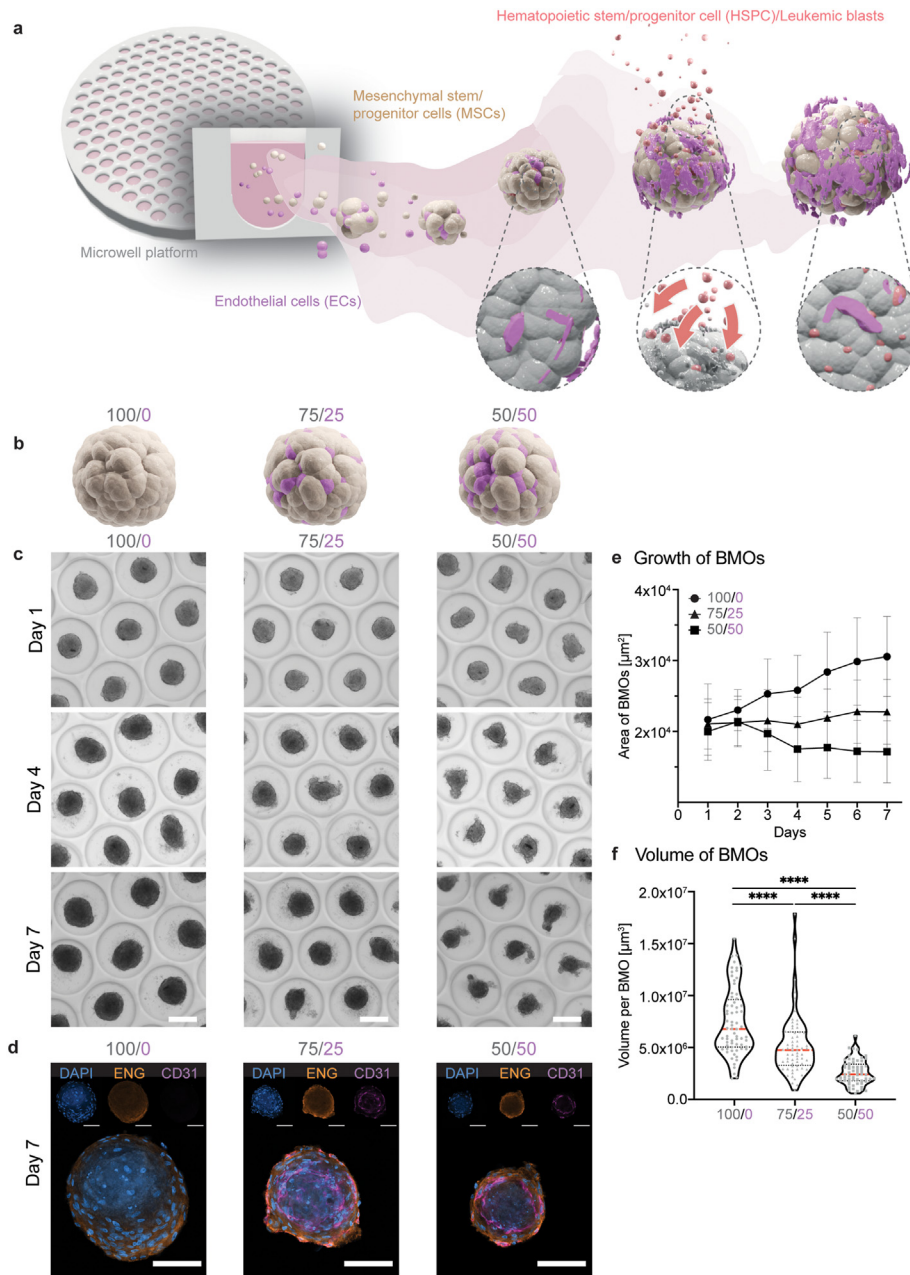


FIG. 1. Formation of bone marrow organoids in a scalable manner. (a) Schematic illustration of the concept of BMOs. Self-aggregation of MSCs (beige) and ECs (magenta) in the microwell platform for the formation of BMOs. After the initial aggregation, HUVECs self-organize to form an endothelial network within the mesenchymal tissue. HSPCs and leukemic blasts (red) are attracted by the presence of multiple BM cell types in a homing-like behavior and take up residence in this BM-mimicking environment. Together with the easily accessible and scalable characteristics of BMOs, they offer a promising *in vitro* 3D model system for BM research and drug candidate screening. (b) Schematic illustration of the aggregation of different ratios of MSCs (beige) and ECs (magenta). The control condition consists of only mesenchymal cells (100/0, left), and the two multi-cell type conditions contain either 25% (75/25, center) or 50% (50/50, right) endothelial cells. (c) Representative brightfield images of BMOs with different cell ratios in the Gri3D platform at different time points (day 1, 4, 7). Scale bar, 200 μm . (d) Representative confocal images of the mesenchymal marker expression endoglin (ENG, orange), the endothelial marker CD31 (magenta), and DAPI (4',6-diamidino-2-phenylindole, blue) marking the cell nuclei in BMOs. The maximum intensity projections of z-stack acquisitions are shown. Scale bar, 100 μm . (e) Growth of BMOs via the analysis of brightfield images by an automated script in Fiji. Analysis of 300–1600 BMOs per condition. Symbols indicate the mean values, and the error bars show the standard deviation. Results of four independent experiments. (f) Spheroids were cultured for seven days, and after fixation, the estimated BMO volume was calculated based on a mask made from the data of ENG expression on the surface of BMOs. Violin plots represent the smoothed distributions, red horizontal lines indicate the medians, and black dotted lines the quartiles. Results of four independent experiments. Statistical analysis by ordinary one-way analysis of variance (ANOVA) with multiple comparisons. **** $p < 0.0001$.

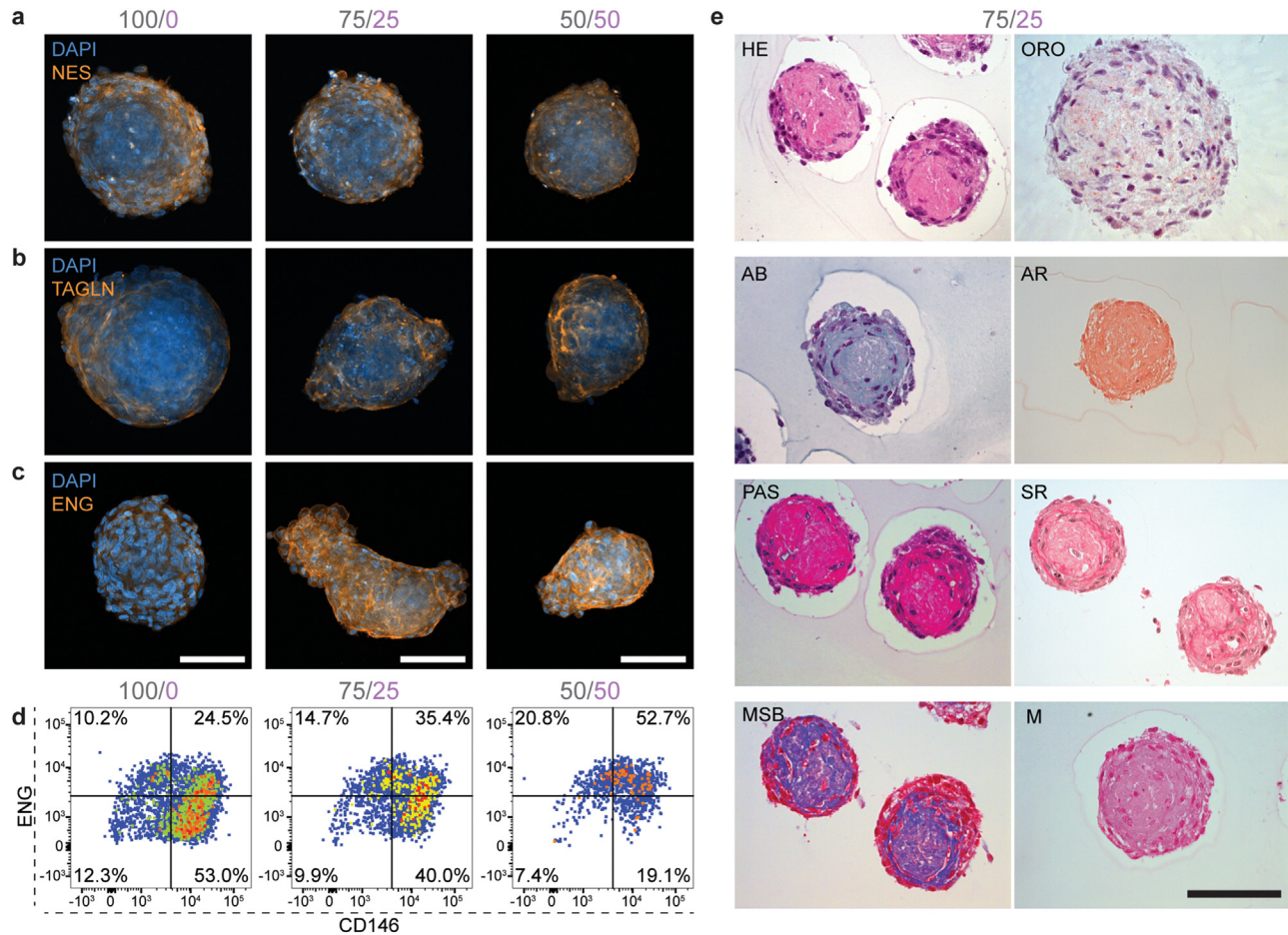


FIG. 2. Self-renewing and differentiated MSCs in bone marrow organoids. (a)–(c) Representative immunofluorescence images of the expression of mesenchymal markers (a) nestin (NES, orange), (b) transgelin (TAGLN, orange), and (c) endoglin (ENG, orange) in BMOs cultured for seven days. Maximum intensity projection of z-stack confocal images. Scale bars, 100 μm . (d) Representative flow diagrams show the percentage of ENG^+ and CD146^+ cells of the $\text{CD71}^-/\text{CD31}^-/\text{CD45}^-$ population in the three different conditions (100/0, 75/25, 50/50). (e) Representative single-plane images of histological stainings of the 75/25 BMO condition cultured for seven days. The morphology is represented by hematoxylin and eosin (HE) staining and Oil Red O (ORO) stained lipid droplets. The Alcian blue (AB) staining marks acidic polysaccharides, such as glycosaminoglycans, and alizarin red (AR) detects calcium deposits. Periodic Acid Schiff (PAS) stains for polysaccharides, Sirius red (SR) stains for collagen, Marius Scarlett Blue (MSB) stains for fibrin, and Miller (M) stains for elastin. Scale bar, 100 μm .

Fig. 2(e)]. Some cell death occurred based on the cleaved Caspase-3 (cCaps3) staining but not exclusively in the eosinophilic core [supplementary material Fig. 2(f)], suggesting that extracellular matrix components are likely secreted to the center of the BMOs. Further histological analyses revealed an abundance of collagen, fibrin, and polysaccharides, such as glycosaminoglycans, which are known extracellular matrix components in the MSCs residency site in natural BM,²⁶ however, there was no elastin accumulation [Fig. 2(e)]. Furthermore, these deposits suggest the commitment of lineage-specific progenitors such as chondrocytes, osteocytes, and adipocytes in BMOs. The presence of the latter is supported by Oil Red O (ORO) staining that revealed the existence of lipid-droplet-bearing adipocytes,²⁷ indicating that a subset of MSCs underwent adipogenic differentiation [Fig. 2(e)]. Next, the detection of SRY-box transcription factor 9 (SOX9), a marker required for the onset of chondrogenesis, supports the partial commitment toward the chondrogenic lineage

[supplementary material Fig. 2(g)].^{26,28} Additionally, the Runt domain-containing transcription factor 2 (RUNX2), a transcription factor regulating osteogenic MSC differentiation,²⁶ was detected in the co-cultures (75/25; 50/50) [supplementary material Fig. 2(h)]. However, no calcium deposits were detected after the seven-day cultivation period in BMOs, indicated by negative alizarin red (AR) staining [Fig. 2(e)]. Overall, this phenotypic analysis shows that the co-culture of MSCs and ECs in BMOs positively influences the maintenance of self-renewing MSCs and simultaneously allows a partial maturation into adipocytes, chondrocytes, and pre-osteoblasts.

Establishment of a self-organized, network-like structure

We further characterized the capacity of the endothelial cells to form network-like structures within the mesenchymal cells by

immunofluorescence analysis. In contrast to aggregates consisting purely of mesenchymal cells, those containing endothelial cells displayed a network structure, evidenced by the general endothelial markers CD31 and CD144 [Fig. 3(a); [supplementary material](#) Figs. 3(a) and 3(b)]. Notably, higher numbers of ECs in the initial culture condition did not result in an increased proportion of CD31⁺-expressing cells [Fig. 3(b); [supplementary material](#) Figs. 3(c) and 3(d)], suggesting that EC survival might depend on the relative number of MSCs in the co-culture. The expression of the endothelial-specific adhesion molecule CD144 within the CD31⁺ population further suggests that endothelial cell–cell contacts are established through surface junctions [[supplementary material](#) Fig. 3(e)]. Whereas active sprouting seems to occur very rarely, indicated by low vascular endothelial growth factor receptor (VEGFR) expression [[supplementary material](#) Fig. 3(f)]. These results demonstrate that ECs can self-organize into an interconnected endothelial network in a 3D co-culture with MSCs.

The complexity of such an endothelial network architecture was visualized and quantified by 3D-rendered and skeletonized data based on CD31 immunofluorescence images [Fig. 3(c); [supplementary material](#) Fig. 3(g)]. This quantification showed that the largest and most complex networks, in terms of number of junctions and branches, were formed in the 75/25 condition [Figs. 3(d)–3(f)]. Subsequently, we analyzed the evolution of the network architecture in this condition over seven days of culture [Fig. 3(g)]. We measured stable network parameters for six days with a slight drop in both network volume and the number of branches and junctions on the seventh day in culture [Figs. 3(h)–3(j)]. Altogether, our finding of a stable, interconnected, network-like structure in dual-cell type bone marrow organoids suggests that this 3D co-culture system partially resembles the vascular niches within the native BM environment.

Bone marrow organoids as a 3D migration assay

Next, we aimed at developing a 3D-*in vitro* assay with these BMOs to investigate the homing ability of human HSPCs [Fig. 4(a)]. To that aim, HSPCs derived from cord blood (CB) samples [see Methods, [supplementary material](#) Fig. 4(a)] were added to three-day-old BMOs with or without an EC network [Fig. 4(a)]. After an additional four days of culture, the number of successfully homed HSPCs within the BMOs was analyzed by immunofluorescence staining [Fig. 4(a)]. This analysis demonstrated the presence of homed HSPCs marked by the pan-hematopoietic marker CD45 within BMOs in all three conditions [Fig. 4(b); [supplementary material](#) Video 2; [supplementary material](#) Fig. 4(b)]. Strikingly, BMOs containing ECs displayed a significantly higher number of homed CD45⁺ HSPCs compared to aggregates consisting purely of mesenchymal cells [Fig. 4(c)]. This result emphasizes the importance of the endothelial compartment in BMOs for modeling HSPC homing behavior *in vitro*. Titration experiments in 75/25 conditions showed that the number of successfully homed HSPCs was dependent on the number of initially seeded cells [Fig. 4(d)]. To further characterize the homing behavior, we analyzed the location of the HSPCs within the BMOs by quantifying their relative distance from the surface of the organoid (D_o) and from the network (D_n) based on the 3D-rendered data [Fig. 4(e)]. Generally, the relative frequency distribution showed that HSPCs migrate, on average, deeper into BMOs than what would be expected when assuming a uniform distribution within the organoid volume (25–30 μm , black dotted line) [Fig. 4(f)]. The observation that ECs are

preferentially arranged on the outer region of BMOs could explain why HSPCs in the dual-cell type BMOs typically reside closer to the organoid surface [Fig. 4(f)]. This observation was confirmed in both dual-cell type conditions, where HSPCs were preferentially found in proximity to the endothelial cells [Fig. 4(g)]. Indeed, over 90% of HSPCs resided closer to the endothelial network structure when compared to the distance expected when generated from a modeled random distribution ($9.8 \pm 4.1 \mu\text{m}$ in 75/25 and $10.0 \pm 6.8 \mu\text{m}$ in 50/50) [Fig. 4(h)]. Additionally, a representative light sheet imaging of a 75/25 BMO provides a 3D animated view of HSPCs in BMOs ([supplementary material](#) Video 3). First, these results show that HSPCs can efficiently migrate inside the organoid structure, and second, the abundance of ECs in BMOs positively influences the migratory behavior of HSPCs in this 3D-homing assay. Overall, BMOs containing the largest EC network (75/25) resulted in the most efficient homing of HSPCs, suggesting that these BMOs provide an optimal environment for such a 3D-migration assay.

Dynamics and mechanism of CD34⁺ HSPC homing

After showing that HSPCs have the potential to migrate inside the BMOs, we addressed the homing dynamics and mechanism of highly enriched HSPCs (CD34⁺ HSPCs, purity around 90%) [Fig. 5(a); [supplementary material](#) Fig. 5(a)]. The CD34⁺ HSPCs were pre-labeled with carboxyfluorescein succinimidyl ester (CFSE) prior to their addition to BMOs (see Methods). Analogous to the previous procedure, 200 CFSE-labeled HSPCs were seeded on fully aggregated BMOs on day 3, and their occurrence within BMOs was analyzed over time by immunofluorescence analysis [Fig. 5(b); [supplementary material](#) Fig. 5(b)]. The first CFSE⁺ cells were already detected within the BMOs 8 h after seeding [Fig. 5(c)]. A constant increase in the number of CFSE⁺ cells was detected during the first 24 h, and a consistent, but slightly lower, increase was observed up until 96 h [Fig. 5(c)]. This migratory behavior resembles *in vivo* HSPC homing behavior, which occurs around 20 h after intravenous injection into the BM of humanized mice.²⁹ Given that CD34⁺ stem cells are known to be a highly quiescent cell population,^{30,31} the detected increase in cell number within BMOs is likely due to an increase in homed cells rather than proliferation. Indeed, a non-bimodal distribution of CFSE intensities over the time course supports this hypothesis [[supplementary material](#) Fig. 5(c)], as the CFSE intensity would decrease by half during each cell division. These results also indicate that efficiently homed CFSE⁺ cells remain within the 3D niche for up to four days.

Thereafter, we asked whether the homing of CD34⁺ HSPCs in BMOs is regulated via the C–X–C motif chemokine ligand 12 (CXCL12)/CXCR4 axis and/or the heparin-binding growth factor pleiotrophin (PTN)-signaling pathway. Both pathways have been found to be crucial for the lodgment, transmigration, and maintenance of HSPCs *in vivo*.^{32–34} The chemokine CXCL12, which is secreted by bone marrow stromal cells, can attract CXCR4-expressing HSPCs to the BM niche.^{35,36} On the contrary, PTN, which is mainly secreted by ECs, regulates the retention of HSCs in the BM niche via the interaction with its transmembrane receptor PTPRZ (Protein Tyrosine Phosphatase Receptor Type Z).³³ We found that CXCR4 was expressed by approximately 15% of the CD34⁺ HSPCs [[supplementary material](#) Figs. 5(d) and 5(e)] and PTN-positive cells were found broadly distributed throughout the BMOs ([supplementary material](#) Figs. 5(f) and 5(g)). To test the involvement of CXCL12–CXCR4 interaction, we selected the CXCR4 antagonist AMD3100 and the C3aR

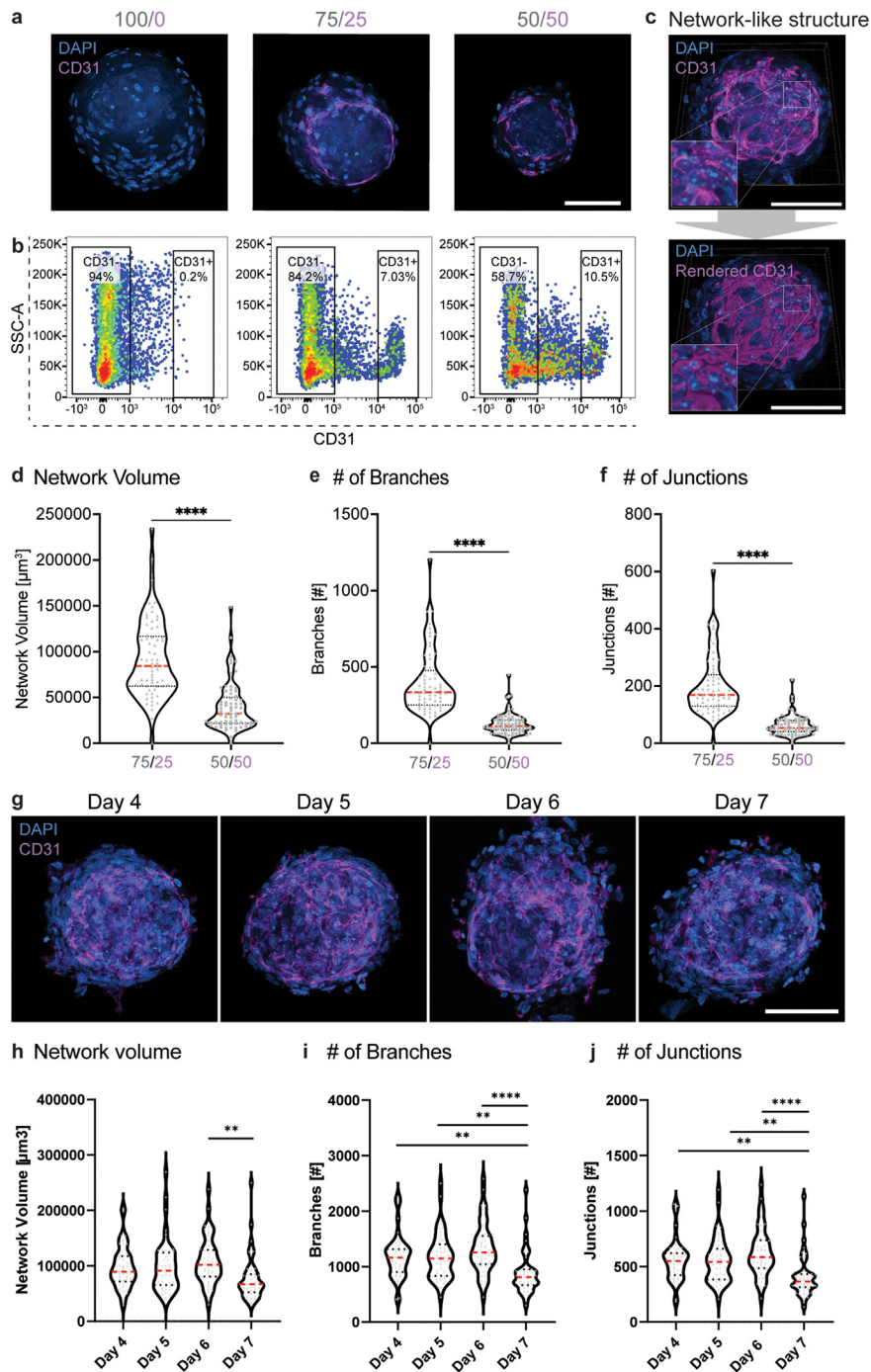


FIG. 3. Establishment of a self-organized, network-like structure. (a) Representative single-plane confocal images of the endothelial marker CD31 (magenta) and DAPI (blue) in different BMOs (100/0, 75/25, 50/50). Scale bar, 100 μm . (b) Representative flow diagrams show the percentage of CD31⁺ cells among all live cells of BMOs from the different conditions (100/0, 75/25, 50/50). (c) 3D visualization (maximum intensity projection) of a z-stack image of CD31 (top panel) and the resulting rendered data (bottom panel) of the self-organized endothelial network-like structure in the BMO condition 75/25. Scale bar, 100 μm . (d)–(f) Quantification of the network architecture by estimating (d) the volume, (e) the number of branches, and (f) junctions. Results from four independent experiments. (g) Representative images of the endothelial marker CD31 in BMOs at day 4–7. Maximum intensity projection of z-stack confocal images. Scale bar, 100 μm . (h)–(j) Quantification of the endothelial network (h) volume, (i) branches, and (j) junctions at different time points (day 4–7). Results of three independent experiments. (d)–(f) and (h)–(j) Violin plots show medians (red dashed line) and quartiles (black dotted lines). Statistical analysis by ordinary one-way ANOVA and multiple comparisons. ** $p < 0.01$; **** $p < 0.0001$.

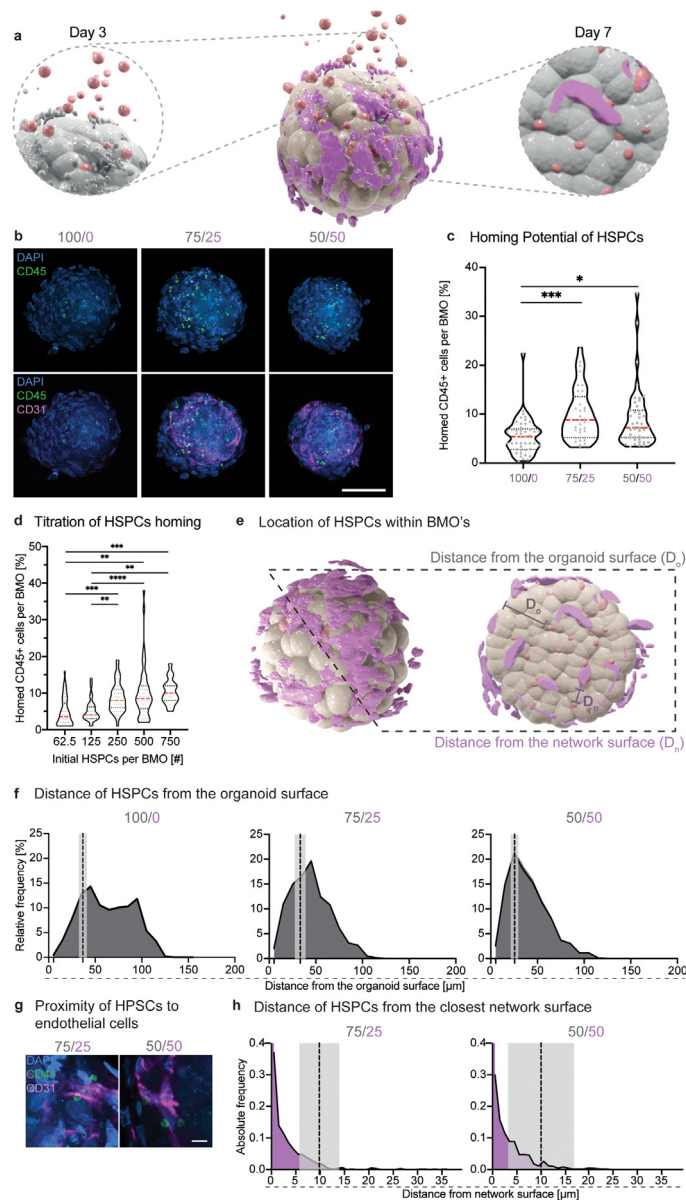


FIG. 4. Bone marrow organoids as a 3D migration assay. (a) Schematic representation of the seeding of HSPCs (red) onto BMOs on day 3 and the subsequent analysis of efficiently homed HSPCs on day 7. (b) Representative confocal images of the HSPCs (CD45, green) and the network (CD31, magenta) in BMOs (DAPI, blue) from the three conditions (100/0, 75/25, 50/50). The maximum intensity projections of z-stack acquisitions are shown. Scale bar, 100 μm . (c) Quantitative analysis of the percentage of homed CD45⁺ cells per BMO normalized to the initial number of seeded HSPCs. Violin plots represent the distributions, red horizontal lines indicate the medians, and black dotted lines the quartiles. Results of four independent experiments. Statistical analysis by ordinary one-way ANOVA and multiple comparisons. * $p < 0.05$; *** $p < 0.001$. (d) Quantitative analysis of the number of homed CD45⁺ cells per BMO (condition: 75/25), derived from different initial seeding numbers of lineage-depleted HSPCs. Violin plots represent the smoothed distributions, red horizontal lines indicate the medians, and black dotted lines the quartiles. Results of three independent experiments. Statistical analysis by ordinary one-way ANOVA and multiple comparisons. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (e) Schematic illustration of the definition of the distance to the organoid (D_o) and network surface (D_n) in BMOs. (f) Relative frequency distributions of the distance of the HSPCs from the organoid surface (D_o) in the different BMO conditions (100/0, 75/25, 50/50). The dashed lines indicate the expected average distance by modeling per condition [100/0: $36.7 \pm 4.2 \mu\text{m}$, 75/25: $33.2 \pm 5.6 \mu\text{m}$, 50/50: $25.4 \pm 4.7 \mu\text{m}$; mean \pm SD (shaded regions)]. Around 79% of HSPCs in the 100/0, 68% in the 75/25, and 73% in the 50/50 condition migrate deeper in BMOs than the expected distance. Results of three independent experiments. (g) Example confocal images (single plane) illustrating proximity of homed HSPCs to the endothelial network. Scale bar, 10 μm . (h) Absolute frequency distributions of the distance of the HSPCs from the endothelial network surface in the multicellular BMOs (75/25 and 50/50). The dashed lines indicate the average expected distance by modeling per condition [75/25: $9.8 \pm 4.1 \mu\text{m}$, 50/50: $10.0 \pm 6.8 \mu\text{m}$; mean \pm SD (shaded region)]. Around 94% of HSPCs in the 75/25 and 92% in the 50/50 condition reside closer to the endothelial structure in the BMO than the expected distance that would result from a uniform distribution. Results of three independent experiments.

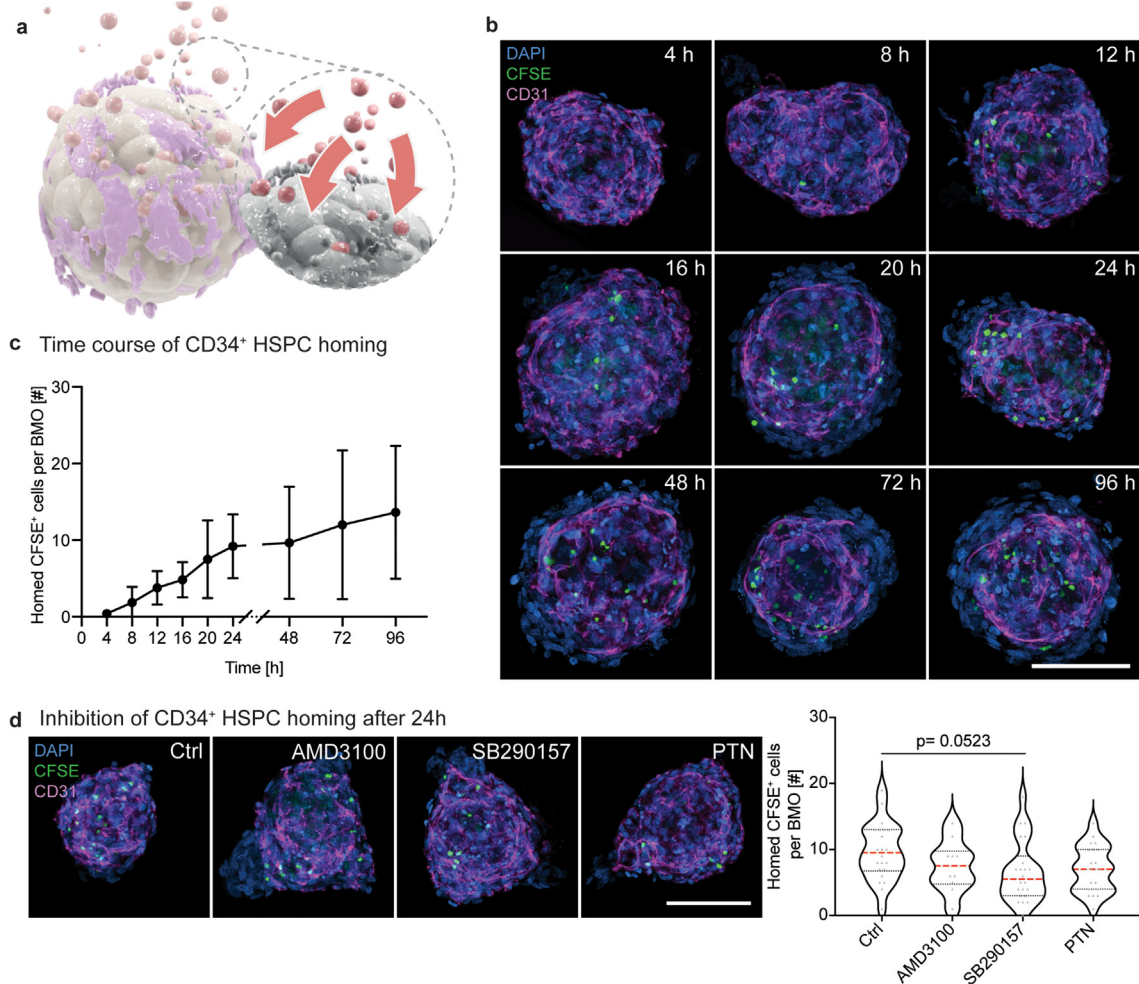


FIG. 5. Dynamics and mechanism of CD34⁺ HSPC homing. (a) Schematic illustration of the homing dynamics of HSPCs (red) into BMOs (magenta/beige/gray). (b) Representative confocal images of CFSE-labeled HSPCs (CFSE, green) and the network (CD31, magenta) within BMOs (DAPI, blue) at 4, 8, 12, 16, 20, 24, 48, 72, and 96 h after seeding of HSPCs. The maximum intensity projections of z-stack acquisitions are shown. Scale bar, 100 μ m. (c) Quantitative analysis of the number of CFSE⁺ cells per BMO over time. Results of two independent experiments. Symbols indicate mean values, and the error bars show the standard deviations. (d) Representative confocal images of the inhibition of homing of HSPCs into BMOs 24 h after seeding (left panel). The purified HSPCs (CFSE, green) are treated with either AMD3100 or SB290157 before seeding on BMOs. In the third condition, BMOs are treated with anti-PTN before seeding of the HSPCs to negatively influence the migration behavior of the HSPCs. The network is marked with CD31 (magenta) and the nuclei with DAPI (blue). The maximum intensity projections of z-stack acquisitions are shown. Scale bar, 100 μ m. The number of homed CFSE⁺ cells is quantified per BMO per treatment (right panel). Violin plots represent the distributions, red horizontal lines indicate the medians, and black dotted lines the quartiles. Results of two independent experiments. Statistical analysis by ordinary one-way ANOVA and multiple comparisons.

antagonist SB290157, both known to negatively influence this interaction.^{37,38} The first two conditions had CD34⁺ stem cells treated with either of these two inhibitors prior to seeding on BMOs, while the third condition included a pretreatment of the BMOs with an anti-PTN antibody. All treatments showed a reduced number of CFSE⁺ cells within BMOs after 24 h with the largest reduction observed in the SB290157 treatment ($p = 0.0523$) [Fig. 5(d)]. These results suggest that the homing of HSPCs in BMOs is partly regulated by the CXCL12–CXCR4 signaling pathway, underlining the potential of the BMO system to model an *in vivo*-like homing behavior of HSPCs *in vitro*.

Bone marrow organoids as a niche for leukemic blasts

Standard *in vitro* toxicology studies often fail to detect drug side effects, which can only be observed in elaborate and expensive *in vivo* studies. Moreover, the poor predictivity of drug responses from *in vitro* screenings has led to many unsuccessful clinical trials due to the fact that cell responses differ based on whether they reside in their (patho-)physiological environment or an oversimplified *in vitro* setting. To showcase the potential value of BMOs for studying leukemia treatments, we tested the capacity of BMOs as a supportive environment for CD34⁺ leukemic blasts. Accordingly, leukemic blast cells were enriched for the CD34⁺ population and labeled with CFSE

[supplementary material Fig. 6(a)]. This enriched population was then seeded on three-day-old BMOs, and the number of internalized cells was assessed over time [Fig. 6(a); supplementary material Fig. 5(b)]. Surprisingly, the first CFSE⁺ cells were detected within BMOs after only 4 h [Fig. 6(b)]. The continuous increase in CFSE⁺ leukemic blast cells within the BMOs, together with a constant CFSE intensity over a 96 h timeframe, suggests an efficient migration of leukemic blasts rather than their proliferation within BMOs [Figs. 6(b) and 6(c)]. Additionally, this rapid migration of leukemic blasts compared to healthy HSPCs correlates with a higher expression of CXCR4 on leukemic blasts [supplementary material Figs. 5(e) and 6(b)]. These results demonstrate that leukemic blasts can efficiently migrate in BMOs. Ultimately, BMOs inhabited by leukemic blasts could be used as an *in vitro* model system to allow large compound screenings on a multicellular entity.

DISCUSSION

Laborious experimental setups are needed to access living BM; thus, it is of high interest to develop *in vitro* assays that provide an easily reachable BM environment for disease modeling. Previous studies have created *in vitro* BM niches to assay HSPC maintenance,

expansion, and differentiation using scaffolds, gels, *in vitro-in vivo* hybrid systems, bioreactors, or microfluidic chips.^{12,20,21,39–44} Instead, the focus of our work was directed toward the development of a self-organized, highly reproducible BM-mimicking platform, which allows for the investigation of stem cell behavior in a scalable manner. Here, we present a dual-cell type BMO system as a novel approach that recapitulates key aspects of human BM, provides a 3D environment for HSPCs, and mimics the HSPC engraftment dynamics *in vitro*. Furthermore, diseased HSPCs, such as CD34⁺ leukemic blasts, showed a similar migration potential, opening the possibility of performing pharmacokinetic and toxicity studies of novel drugs within such a 3D *in vitro* assay prior to *in vivo* studies. These BMOs were built by co-aggregating human MSCs and ECs, which self-organize into spherical aggregates containing an endothelial network structure. Interestingly, within this 3D co-culture system, a population of MSCs was maintained while other MSCs partially differentiated toward chondrocytes, adipocytes, and osteoblasts, indicated by the deposition of extracellular matrix proteins and specific transcription factors. Calcium deposits were not detected, probably due to the short culture period of these BMOs, considering standard osteogenic differentiation protocols of this process take three or more weeks.⁴⁵ The adipo-

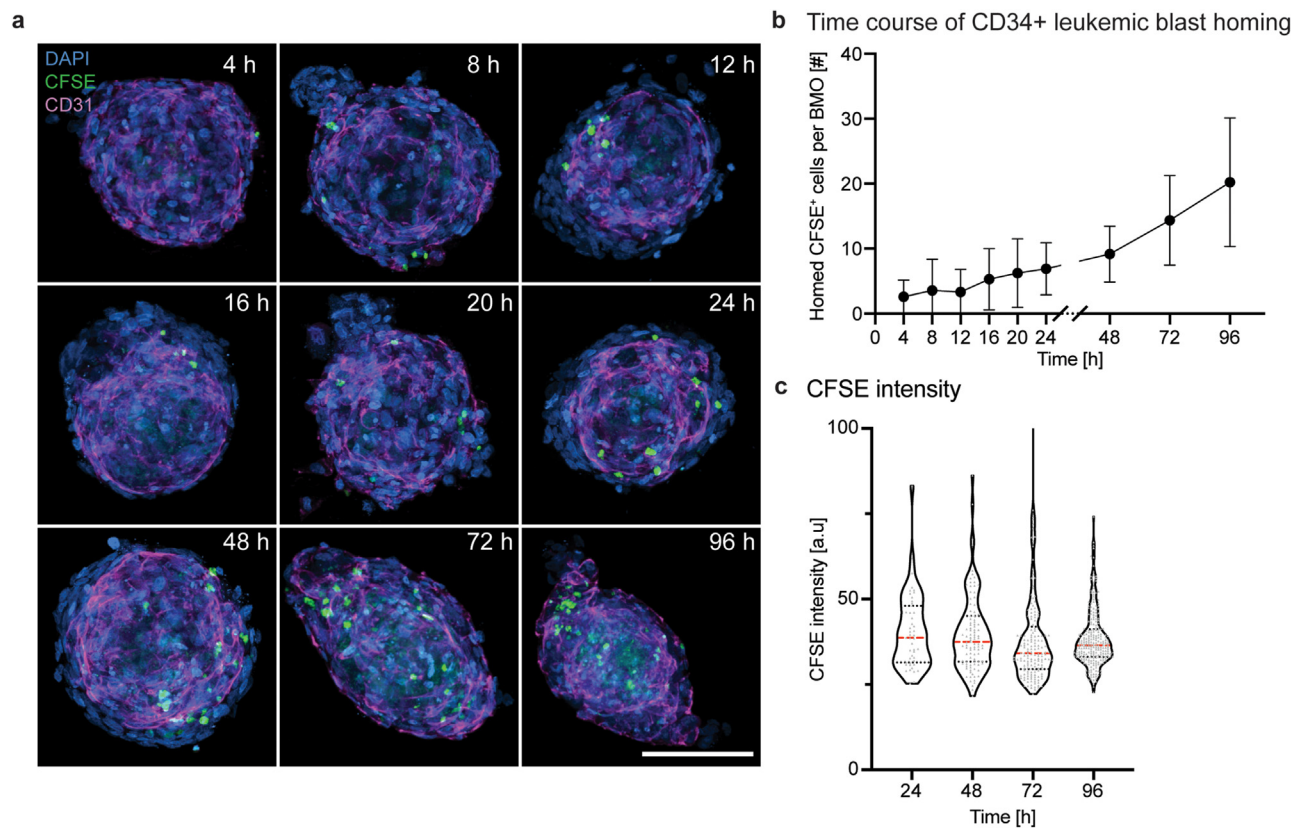


FIG. 6. Bone marrow organoids as a niche for leukemic cells. (a) Representative confocal images of leukemic blast cells (CFSE, green) and the network (CD31, magenta) within BMOs (DAPI, blue) at 4, 8, 12, 16, 20, 24, 48, 72, and 96 h after their addition. The maximum intensity projections of z-stack acquisitions are shown. Scale bar, 100 μm . (b) Quantitative analysis of the number of CFSE⁺ cells per BMO over time. Results of two independent experiments. Symbols indicate mean values, and the error bars show the standard deviations. (c) Quantification of the CFSE intensity of individual homed leukemic stem cells (LSCs) over 96 h. Representative data from one out of three independent experiments. Violin plots represent the smoothed distributions with individually analyzed CFSE⁺ cells represented as gray dots. Dotted horizontal lines indicate the medians.

osteogenic balance of MSCs, which we detected in our system, mimics an essential part of the natural niche environment that is known to be crucial for hematopoiesis.^{3,46} Additionally, the deposition of extracellular matrix proteins by MSCs in BMOs could serve as a reservoir for growth factors and provide non-cellular ligands, which is known to be a critical process for the migration and homing of HSPCs in the BM.⁴⁷ Overall, our results confirmed previously described mesenchymal 3D cultures,¹⁹ including the maintenance of the MSC fate, the deposition of extracellular matrix proteins, and as demonstrated for the first time, the presence of differentiated mesenchymal phenotypes within dual-cell type BMOs.

Importantly, most studies presenting *in vitro* BM systems used scaffolds,^{18,48} gels,^{25,42,49–51} or microfluidic devices^{20,21,41,43,52} to obtain complex endothelial networks *in vitro*. We demonstrate here that ECs have the potential to self-organize in a complex architectural network within mesenchymal cells without additional support, reminiscent of the organization found in the vascular BM. The intricate balance between the two cell types is highlighted by the finding that elevated numbers of starting ECs did not result in more ECs within BMOs nor formed a larger network. These results suggest that EC survival and network formation are limited by the secretion of cytokines and/or the disposition of an adhesive substrate by mesenchymal cells in BMOs. Additionally, we observed at day 7 a reduction of the endothelial network in BMOs. Therefore, further medium optimization are necessary to establish a long-term 3D culture system for BMOs. One possibility could be to switch around day 6 to a “maintenance” medium to sustain (1) low proliferation and minimal matrix deposition in the mesenchymal part and (2) a stable endothelial network for long-time experiments. Overall, we hypothesize that a further characterization of the dependencies on various medium components, nutrition availability, or donor origin of the two cell types will ultimately help to refine conditions enabling BMO maintenance for longer times.

Intriguingly, a homing-like behavior of HSPCs was observed when these stem cells were added to fully formed BMOs, and the migration capacity of these stem cells was positively influenced by the abundance of ECs. *In vivo* studies have shown that such cell–cell interactions are essential for the migration, mobilization, and maintenance of HSPCs within BM.²³ Additionally, it is hypothesized that the first interaction during the homing of HSPC in the BM is with the sinusoidal endothelium.⁵⁴ Therefore, our findings that HSPCs migrated more efficiently in BMOs containing a larger network-like structure, as well as the close proximity between the two cell types, suggest that our 3D system mimics essential parts of such cellular interactions found in natural BM.^{23,34} Further investigation of the migration process of HSPCs in BMOs proposed a partial regulation of the CXCL12–CXCR4 signaling pathway, which is known to be the main regulator for human stem cell homing.³⁵ Only a partial blockage was achieved through the CXCL12–CXCR4 axis, suggesting the involvement of additional signaling pathways. In murine models, evidence was found that CD82, a tetraspanin, is also involved in the regulation of this process.⁵³ Thus, this BMO system could be used as an *in vitro* tool to investigate such cell–cell interactions and molecular signaling pathways of the human HSPC–niche environment. In this work, we showed that BMOs provide a BM-mimicking environment that recapitulates natural HSPC homing behavior. However, the preservation of the HSPC capacity still needs to be assessed through xenotransplantation assays in future studies.⁵⁴

Most importantly, we found that CD34⁺ leukemic blasts have the potential to efficiently migrate and reside in BMOs, similar to healthy HSPCs. The high expression of CXCR4 on CD34⁺ leukemic blasts suggests that their migration capacity is also regulated over the CXCL12–CXCR4 axis. This hypothesis is supported by the evidence that the CXCL12–CXCR4 signaling pathway is crucial for leukemic blast survival as well as for *in vivo* homing and repopulation of the healthy hematopoietic niche.^{55,56} It is known that the vascular density is increased in the AML marrow, while the role of the sinusoidal endothelium on leukemic blasts is still not understood. Thus, our BMOs, which contain a complex endothelial-like network, provide an *in vitro* environment to investigate and understand such endothelial–leukemia cell interactions in future studies. Additionally, this complex *in vitro* BM environment opens up exciting perspectives toward new understandings of clinically relevant mechanisms of resistance as well as of the variability of leukemic blast responses to potential cancer therapeutics.

Overall, we believe that by using the self-assembling potential of MSCs, ECs, and HSPCs/leukemic blasts in 3D, the natural as well as the diseased BM niche is mimicked more precisely than when these interactions are guided in other models (e.g., microfluidic devices). In-depth analysis of the secreted cytokine cocktail derived from dual-cell type BMOs could help to decipher the interplay between the niche and HSPCs/leukemic blasts behavior. Furthermore, the relevance of our system may be enhanced through the addition of even more niche-like cells such as perivascular,⁵⁷ osteoblastic,^{58,59} or nerve⁶⁰ cells.

In conclusion, an *in vitro* assay of next-generation BMOs provides an experimental tool for questions related to basic BM niche biology such as specific cell–cell interactions, homing behavior, or the maintenance of HSPCs and leukemic blasts. Furthermore, the use of BMOs as a testing ground prior to focused *in vivo* experimentation could reduce the number of experimental animals, and thereby, reduce overall costs. The advantages of this system over standard *in vitro* assays are (i) the presence of multiple BM cell types, which creates an *in vivo*-mimicking environment for HSPCs and leukemic blasts, (ii) the 3D context, allowing for systematic searches for dosing regimens, which could grant better prescreening data for future *in vivo* studies, and (iii) the potential to investigate patient-to-patient variability in terms of drug resistance. We additionally believe that the reproducibility and scalability of the Gri3D microwell platform meets crucial requirements for various applications of BMOs from large-scale compound screenings to patient-specific tests.⁶¹ In summary, the multi-cell type 3D BMOs represent an innovative preclinical model for recapitulating hematopoietic and leukemic stem cell dynamics in a complex BM-like niche environment.

METHODS

Cell sources

Human samples were received from consenting donors from the University Hospital Zurich and the Geneva University Hospital in compliance with Swiss law. Frozen vials of MSCs were purchased from Lonza or received from the University Hospital Zurich. HUVECs were received as frozen vials from the University Hospital Zurich. Umbilical cord blood (UCB) was collected from consenting donors from the University Hospital Zurich. BM or peripheral blood (PB) samples from AML patients at diagnosis were obtained with informed consent and approved by the local ethics committee

(Medical Ethics Committee of the Canton Geneva; Study No. 2020-00176). The diagnosis and classification of leukemic samples was performed according to the WHO 2016 classification. The mononuclear fraction of CB and AML cells were isolated by using a Ficoll density centrifugation with Lymphoprep (Axonlab, 1114545) in Leucosep tubes (Greiner bio-one, 227290). Prior to culturing or freezing AML cells, the mononuclear fraction was further enriched for CD34⁺ cells using the UltraPure CD34 Microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany, 130-100-453) and magnetic-activated cell-sorting separation columns (Miltenyi Biotec, 130-042-201). The mononuclear CB samples were cryopreserved with serum-free freezing medium (FILOCETH⁶²) and stored in liquid nitrogen.

Magnetic depletion and enrichment of cord blood and AML cells

The cryopreserved CB vials were thawed and diluted dropwise with IMDM plus GlutaMAX (1×, Gibco, 31980-030) containing 0.5% bovine serum albumin (BSA) Fraction V (Gibco, 15260-037) and 10 μg/ml DNase I (Roche, 11284932001). The cryopreserved AML cells were thawed by adding dropwise StemSpan SFEM II medium (StemCell Technologies, 09605) supplemented with 1% penicillin/streptomycin (P/S) (Gibco, 15140-122). The dead cell removal kit (Miltenyi Biotec, 130-090-101) was used to remove apoptotic and dead cells via magnetic depletion according to the indicated instructions. The CB cells were lineage depleted with the human lineage cell depletion kit (Miltenyi Biotec, 130-092-211), which contains the following biotin-conjugated monoclonal antibodies, CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a (Glycophorin A). The Lin⁻ fraction of the CB cells and the AML cells were further enriched for CD34 with the human CD34 MicroBead Kit UltraPure (Miltenyi Biotec, 130-100-453), which contains microbeads conjugated to monoclonal mouse anti-human CD34 antibodies (isotype: mouse IgG1). For the depletion or enrichment, the cells were resuspended in phenol red-free IMDM (1×, Gibco, 21056-023) or phosphate-buffered saline (PBS) (pH 7.4, 1×, Gibco, 10010-015) containing 0.5% BSA Fraction V, 2 mM EDTA (pH 8.0, Gibco, 15575-020), 1% P/S and optionally 20 mM Hepes (Gibco, 15630-056). The enrichment or depletion was performed according to the manufacturer's protocols and run over the LS or MS columns (Miltenyi Biotec, 130-042-401 or 130-042-201, respectively) depending on the total cell number.

Flow cytometry

The purity check of the lineage depletion or enrichment of HSPCs was performed by staining with the following monoclonal directly conjugated antibodies CD45-PECy5 or CD45-AF700, CD34-BV421 or CD34-PE, CD38-APC and optionally with the biotin lineage depletion cocktail and streptavidin-AF488 (supplementary material Table 1). The CD34⁺ AML cell purity were assessed by staining cells with anti-human CD34-PE-Vio770 and CD45-VioGreen (supplementary material Table 1). To assess the expression levels of CXCR4, the CD184-BV605 monoclonal antibody was used (supplementary material Table 1). The single cells from the digested BMOs were stained and analyzed with the following monoclonal directly conjugated antibodies: CD71-AF700, CD31-APCeFluor780 or AF700, CD105(ENG)-FITC, CD146-BV711, VEGFR-PE, CD144-PECy7, CD45-PECy5, CD34-BV421, and CD38-APC (supplementary material Table 1).

For live/dead cell discrimination, 4',6-diamidino-2-phenylindole (DAPI, Sigma, D9542), propidium iodide (PI, Sigma, 81845), or 7-Amino-Actinomycin D (7-AAD, Beckman Coulter, B88526) was added to the cell suspensions. UltraComp eBeads (invitrogen, 01-2222-42) were used to create single color controls compensation. The LSRII, LSR Fortessa (Becton Dickinson), or Navios 10-color (Beckman Coulter) flow cytometers were used to analyze the samples. Data were analyzed in Flowjo (Version 10.5.3) or Kaluza (Analysis Version 2.1), and fluorescence minus one controls (FMOs) were used to set the gates (data included in supplementary material figures).

2D cell culture

Human MSCs were expanded on 2D flasks in αMEM (Gibco, 12571-063) supplemented with 10% fetal bovine serum (FBS, Gibco, 10500-064), 2 mM GlutaMax, 1% P/S and 1 ng/ml human fibroblast growth factor 2 (FGF2) (Peprotech, 100-18B). When the MSCs reached 80%–90% confluency, the cells were detached with 0.25% Trypsin (Gibco, 25200-072) and partially re-plated in expansion medium or used for the 3D culture. For cryopreservation, the MSCs were stored in 90% FBS containing 10% dimethyl sulfoxide (DMSO) (Sigma, D2438).

HUVECs were expanded on 2D flasks in Endothelial Cell Growth Medium-2 (EGM-2, Lonza, CC-3162) or Endothelial cell growth medium, FCS-kit, advanced (ECGM, Provitro 211 1101). If the confluency reached around 80%–90%, the HUVECs were detached with TrypLETM Express (Gibco, 12605-028) and partially re-plated in the expansion medium or used for the 3D culture. The cryopreservation was done in a medium containing 60% EGM-2 or ECGM, 30% FBS and 10% DMSO.

CD34⁺ leukemic blasts were cultured in StemSpanTM SFEM II serum-free medium (STEMCELL Technologies, 09605) supplemented with the StemSpan CD34⁺ Expansion supplement (STEMCELL Technologies, 02691) and 1% P/S. Cells were initially plated in 96-well culture plates at a concentration in the range of 5–10 × 10⁴ cells/ml and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. Complete medium change was performed every third day. Cultures were maintained for several months. All cells were cryopreserved in liquid nitrogen until usage.

3D cell culture

For the 3D culture of MSCs and HUVECs, cells were seeded in different ratios in the Gri3D cell culture platform (SUN bioscience, Gri3D-24-S-24P, Gri3D-96-S-24P).²² The 24 or 96 well plate format of the Grid3D platform was used. Each well contained an array with 121 PEG μ-wells with a diameter of 400 μm. For the control condition (100/0), 500 MSCs were seeded per μ-well. For the coaggregation of MSCs and HUVECs, either 250 MSCs and 250 HUVECs (50/50) or 375 MSCs and 125 HUVECs (75/25) were seeded per μ-well. The cells were cultured in previously published medium^{19,24} that contains the following components: Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Gibco, 31331-028) and human endothelial SFM (Gibco, 11111-044) supplemented with 0.1 mM 2-mercaptoethanol (50 mM, Gibco, 31350-010), 1× non-essential amino acids (Gibco, 11140-035), 1× N2 (Gibco, 17504-001), 1× B27 supplements (Gibco, 17504001), 1% P/S, recombinant human fibroblast growth factor 2 (hFGF-2, Peprotech, 100-18B), recombinant human

epidermal growth factor (hEGF, 20 ng/ml, Chimerigen Laboratories, CHI-HF-210EGF), recombinant human platelet derived growth factor (hPDGF-AB, 20 ng/ml, Merck, GF106), recombinant human oncostatin M (hOSM, 20 ng/ml, Gibco, PHC5015) and recombinant human insulin-like growth factor 1 (hIGF-1, 40 ng/ml; Peptotech, 100-11), and 15% chick embryo extract (CEE).^{19,24} CEE was produced according to published protocols.^{63,64} The day of seeding MSCs and HUVECs in μ -wells is referred to as day 0. The full medium was replaced every second or third day. The addition of the CD34⁺ HSPCs was performed on day 3 when compact BMO were typically formed. 62.5 to 750 human lineage-depleted (Lin⁻) CB cells, CD34-enriched (CD34⁺) HSPCs, or CD34⁺ AML blasts were seeded per BMO on day 3. The CD34⁺ HSPCs and CD34⁺ AML blasts were labeled with the CFSE cell division assay kit (Cayman, 10009853), according to the manufacturer's instructions prior to their seeding on BMOs. Every 4 h for the initial 24 h and then after 48, 72, and 96 h BMO were collected for further analysis. If the CD34⁺ cells were added to the BMOs, the collection was performed using a FACS strainer (Falcon, 352235) to remove non-homed cells. For the inhibition of the homing potential, the CD34⁺ HSPCs were treated with 100 μ M of AMD3100 octahydrochloride (Tocris, 3299) or with 8 μ M of SB290157 (Sigma, SML1192) for 45 min at 37 °C. The anti-PTN antibody (R&D, AF-252) was added in a 1/25 dilution to the culture medium of the BMOs for 45 min at 37 °C. For the cytometric analysis of the surface markers, the BMOs were digested with PBS containing 3 mg/ml collagen IV (Gibco, 17104-019), 4 mg/ml Dispase (Gibco, 17105-041) and 2 mg/ml DNase I (Roche, 11284932001) to obtain a single cell suspension.

Immunostaining

The collected BMOs were fixed with 2% paraformaldehyde (PFA, ThermoFischer Scientific, 15434389) for 1 h and permeabilized with 0.3% Triton-X-100 (Sigma, T8787) in PBS for 3 h at RT. Depending on the secondary antibody either 1% Albumin Fraction V (AppliChem, A1391.0500) or 10% donkey-serum in PBS 0.01% Triton-X-100 as blocking solution was used at 4 °C overnight. After the blocking step, the following primary antibodies were used for the immunostainings (supplementary material Table 2) in combination with the below listed secondary antibodies (supplementary material Table 3). DAPI was used to mark the nuclei.

Microscopy

For the growth of the BMOs, brightfield images of the Grid3D array were acquired every day or every second day on a Nikon Ti microscope with a 4 \times objective (N.A. = 0.13, air) and automated stitching of tiles to form large images. Confocal images for qualitative representation were acquired on an inverted LSM700 microscope (Zeiss) with a 20 \times objective (N.A. = 0.8, air). The pixel size was set to 0.16 μ m, and a z-step of 3 μ m, pinhole set a 36.8 μ m (equivalent DAPI: 1.28 A.U. = 1.8 μ m, A647: 0.91 A.U. = 2.3 μ m, A488: 1.13 A.U. = 2.2 μ m, A568: 1.09 A.U. = 2.2 μ m) was used. Confocal images, which were analyzed with a custom script (see Methods, Image analysis), were acquired on an upright LSM710 microscope (Zeiss) or an upright SP8 microscope (Leica) with 20 \times objectives (N.A. = 0.8, air and N.A. = 0.75, air, respectively). For both, the pixel size was set to 0.71 μ m and a z-step of 0.5 μ m was used. For the LSM710, the pinhole was set to 30.4 μ m (equivalent DAPI and

A647: 1.15 A.U. = 1.6 μ m; A488: 0.98 A.U. = 1.7 μ m; A568: 0.86 A.U. = 1.7 μ m) and for the SP8, the pinhole was set to 47.5 μ m (equivalent DAPI: 0.87 A.U. = 0.7 μ m; A488: 0.75 A.U. = 0.8 μ m; A568: 0.65 A.U. = 0.8 μ m; A568: 0.58 A.U. = 0.9 μ m). The histology sections were acquired with an upright DM5500B (Leica). The color camera CMOS DMC 2900 was used to acquire the images with a 10 \times (N.A. = 0.3, air), 20 \times (N.A. = 0.7, air), or 40 \times (N.A. = 0.5–1.0, oil) objective.

Image analysis

Growth of BMOs was analyzed in Fiji (2.0.0-rc-69/1.52n) with a custom script, which automatically detects the BMO area by thresholding and particle analysis within the array on brightfield images. The Matlab EasyXT-User Interface [1] for Imaris was used to detect the spheroid and the network as surface objects and to detect the CD45-positive or CFSE-traced cells as spots or surfaces objects. The whole organoid surface was detected using smoothed ENG or DAPI signal and the endothelial network based on the CD31 staining. The mask channel for the individual detected surfaces was generated for further analysis.

For the network architecture, a Fiji [2] custom script was used to extract the endothelial network mask from the Imaris image, skeletonized the data and analyzed the skeleton.⁶⁵ The script relies on the "BIOP Basics" [3] ActionBar to facilitate processing.

- [1] EasyXT (<https://github.com/BIOP/EasyXT>) and EasyXT-GUI (<https://github.com/BIOP/EasyXT-GUI>)
- [2] FIJI (<https://www.fiji.sc>)
- [3] BIOP Basics (https://c4science.ch/w/bioimaging_and_optics_platform_biop/image-processing/imagej_tools/biop-basics/)

For the estimated BMO volume, a FIJI custom script that extracts the BMO mask made in Imaris was used, makes a z-projection, and runs "Analyze particles" with the "fit ellipse" option. From the measurements, it gets the major and minor radii and calculates an estimation of the volume with the formula: $Vol = \frac{4}{3} * \pi * Radius_{majorAxis} * Radius_{minorAxis}^2$. Customized analysis was performed to measure the distances from the cells to the organoid surface and to the endothelial network, respectively. Using EasyXT in Matlab, new channels were generated in Imaris whose voxel's intensities equal the 3D-distance toward the above rendered organoid and endothelial network surfaces, respectively. For each detected cell, the distance toward the organoid or the network surface was approximated by the average intensity of the generated distance channels within the cell volume. To give a reference, the expected average distances were modeled per organoid by averaging the intensities of the distance channels within the whole organoid volume (for the surface-distance modeling) or the network volume-cleared organoid volume (for the network-distance modeling).

Histology

BMOs were collected from the array as described above and fixed with 2% or 4% PFA or 10% Formalin (Sigma, HT501128) overnight at 4 °C. For paraffin sectioning, the BMOs were embedded in a drop of HistoGel (ThermoFisher Scientific, HG-4000-012). The dehydration of the hardened HistoGel drops was done in the automated dehydration machine (Sakura, VIP6). Afterwards, the samples were embedded in paraffin (Merck, 1.11609.2504) according to standard histological

procedures, and 4 μm thick sections of the paraffin blocks were cut on the microtome (Leica, HM325). The paraffin sections were stained with Hematoxylin–Eosin (HE) to assess general morphology. Alcian blue (AB) at pH 2.5 was used to stain mucus, Alizarin Red (AR) for calcium deposits, Periodic Acid Schiff (PAS) for polysaccharides, Sirius Red (SR) stains collagen, Marius Scarlett Blue (MSB) is for fibrin, and Miller (M) stains for elastin.

For the cryosections, the BMOs were placed in an increasing sucrose solution (7.5% for 1 h, 15% for 1 h and 30% O/N; Sigma, S1888) for improved cryoprotection. Afterwards, the BMOs were embedded in a drop of 7.5% gelatin solution (Sigma, G1890) with phosphate buffer (Sigma, 71505 and S7907) 0.12 M and 15% sucrose and placed on an organoid embedding sheet (StemCell Technologies, 68579A). The drop of gelatin was left to harden at 4 °C for 15 min. The gelatin drop was placed in a mold half-filled with gelatin (Sigma, G2500) or cryomatrix (Thermo Scientific, 6769006). The block was slowly frozen in isopentane (VWR, 24872.298) cooled down in a bath of ethanol (Fischer Chemical, E/0600DF/15) and dry ice to -65 to -70 °C. The cryoblocks were cut into 8 μm thick sections on the cryostat (Leica, 1850 or 1950). Oil Red O staining was used to specifically highlight lipids.

Statistical analysis

The according statistical analysis is indicated in each figure's description. Student's t-tests were used to compare two datasets, while ordinary one-way ANOVA with multiple comparisons was used for a group of data. All datasets are represented with mean, median, and standard deviations or median quartiles as indicated. Statistical significance was set at a p-level of less than 0.05.

SUPPLEMENTARY MATERIAL

See the [supplementary material](#) for Figs. 1–6, [supplementary material](#) videos 1–3, and [supplementary material](#) Tables 1–3.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Ethics Approval

Ethics approval for experiments reported in the submitted manuscript on animal or human subjects was granted. BM or peripheral blood (PB) samples from AML patients at diagnosis were obtained

with informed consent and approved by the local ethics committee (Medical Ethics Committee of the Canton Geneva; Study No. 2020-00176).

Author Contributions

Sonja Giger: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Writing – original draft (equal); Writing – review & editing (equal). **Moritz Hofer:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Visualization (equal); Writing – original draft (equal); Writing – review & editing (equal). **Marijana Miljkovic-Licina:** Data curation (supporting). **Sylke Hoehnel:** Conceptualization (supporting). **Nathalie Brandenburg:** Conceptualization (supporting). **Romain Guiet:** Formal analysis (supporting); Methodology (equal); Software (lead). **Martin Ehrbar:** Resources (supporting). **Esther Kleiner:** Resources (equal). **Katharina GEGENSCHATZ-SCHMID:** Resources (supporting). **Thomas Matthes:** Supervision (supporting); Writing – original draft (supporting). **Mathias P. Lutolf:** Conceptualization (equal); Formal analysis (equal); Funding acquisition (equal); Project administration (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal).

DATA AVAILABILITY

The data that support the findings of this study are available within the article and its [supplementary material](#).

REFERENCES

- Q. Wei and P. S. Frenette, “Niches for hematopoietic stem cells and their progeny,” *Immunity* **48**, 632–648 (2018).
- C. Nombela-Arrieta and M. G. Manz, “Quantification and three-dimensional microanatomical organization of the bone marrow,” *Blood Adv.* **1**, 407–416 (2017).
- I. Beerman, T. C. Luis, S. Singbrant, C. Lo Celso, and S. Méndez-Ferrer, “The evolving view of the hematopoietic stem cell niche,” *Exp. Hematol.* **50**, 22–26 (2017).
- K. Szade *et al.*, “Where hematopoietic stem cells live: The bone marrow niche,” *Antioxid. Redox Signal.* **29**, 191–204 (2018).
- J. Saultz and R. Garzon, “Acute myeloid leukemia: A concise review,” *J. Clin. Med.* **5**, 33 (2016).
- J. Versluis *et al.*, *The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies* (Springer, 2018), pp. 1–702.
- E. A. Griffiths, H. E. Carraway, N. S. Chandhok, and T. Prebet, “Advances in non-intensive chemotherapy treatment options for adults diagnosed with acute myeloid leukemia,” *Leuk. Res.* **91**, 106339 (2020).
- W. Grey *et al.*, “Modeling the human bone marrow niche in mice: From host bone marrow engraftment to bioengineering approaches,” *J. Exp. Med.* **215**, 729–743 (2018).
- M. Ghasemi and A. R. Dehpour, “Ethical considerations in animal studies,” *J. Med. Ethics Hist. Med.* **2**, 12 (2009).
- C. Lee-Theedieck, N. Rauch, R. Fiammengo, G. Klein, and J. P. Spatz, “Impact of substrate elasticity on human hematopoietic stem and progenitor cell adhesion and motility,” *J. Cell Sci.* **125**, 3765–3775 (2012).
- A. Roch *et al.*, “Single-cell analyses identify bioengineered niches for enhanced maintenance of hematopoietic stem cells,” *Nat. Commun.* **8**, 1–22 (2017).
- J. S. Choi, B. P. Mahadik, and B. A. C. Harley, “Engineering the hematopoietic stem cell niche: Frontiers in biomaterial science,” *Biotechnol. J.* **10**, 1529–1545 (2015).
- G. Rossi, A. Manfrin, and M. P. Lutolf, “Progress and potential in organoid research,” *Nat. Rev. Genet.* **19**, 671–687 (2018).
- M. Simian and M. J. Bissell, “Organoids: A historical perspective of thinking in three dimensions,” *J. Cell Biol.* **216**, 31–40 (2017).

- ¹⁵A. Xavier da Silveira dos Santos and P. Liberali, "From single cells to tissue self-organization," *FEBS J.* **286**, 1495–1513 (2019).
- ¹⁶J. Lee, D. Heckl, and B. Parekkadan, "Multiple genetically engineered humanized microenvironments in a single mouse," *Biomater. Res.* **20**, 19 (2016).
- ¹⁷L. C. Martine *et al.*, "Engineering a humanized bone organ model in mice to study bone metastases," *Nat. Protoc.* **12**, 639–663 (2017).
- ¹⁸P. E. Bourguine *et al.*, "In vitro biomimetic engineering of a human hematopoietic niche with functional properties," *Proc. Natl. Acad. Sci.* **115**, E5688–E5695 (2018).
- ¹⁹J. Isern *et al.*, "Self-renewing human bone marrow mesospheres promote hematopoietic stem cell expansion," *Cell Rep.* **3**, 1714–1724 (2013).
- ²⁰D. B. Chou *et al.*, "On-chip recapitulation of clinical bone marrow toxicities and patient-specific pathophysiology," *Nat. Biomed. Eng.* **4**, 394 (2020).
- ²¹D. E. Glaser *et al.*, "Organ-on-a-chip model of vascularized human bone marrow niches," *Biomaterials* **280**, 121245 (2020).
- ²²N. Brandenburg *et al.*, "High-throughput automated organoid culture via stem-cell aggregation in microcavity arrays," *Nat. Biomed. Eng.* **4**, 863 (2020).
- ²³J. P. Lévesque, F. M. Helwani, and I. G. Winkler, "The endosteal osteoblastic niche and its role in hematopoietic stem cell homing and mobilization," *Leukemia* **24**, 1979–1992 (2010).
- ²⁴S. Méndez-Ferrer *et al.*, "Mesenchymal and haematopoietic stem cells form a unique bone marrow niche," *Nature* **466**, 829–834 (2010).
- ²⁵P. Au, J. Tam, D. Fukumura, and R. K. Jain, "Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature," *Blood* **111**, 4551–4559 (2008).
- ²⁶T. Assis-Ribas, M. F. Furni, S. M. B. Winnischofer, M. C. Sogayar, and M. Trombetta-Lima, "Extracellular matrix dynamics during mesenchymal stem cells differentiation," *Dev. Biol.* **437**, 63–74 (2018).
- ²⁷M. C. Vemuri, L. G. Chase, and M. S. Rao, *Mesenchymal Stem Cell Assays and Applications*, Methods in Molecular Biology (Springer, Clifton, NJ, 2011), Vol. 698.
- ²⁸V. Lefebvre and M. Dvir-Ginzberg, "SOX9 and the many facets of its regulation in the chondrocyte lineage," *Connect. Tissue Res.* **58**, 2–14 (2017).
- ²⁹M. Takahashi *et al.*, "Comprehensive evaluation of leukocyte lineage derived from human hematopoietic cells in humanized mice," *J. Biosci. Bioeng.* **113**, 529–535 (2012).
- ³⁰E. Laurenti *et al.*, "CDK6 levels regulate quiescence exit in human hematopoietic stem cells," *Cell Stem Cell* **16**, 302–313 (2015).
- ³¹D. J. H. F. Knapp *et al.*, "Dissociation of survival, proliferation, and state control in human hematopoietic stem cells," *Stem Cell Rep.* **8**, 152–162 (2017).
- ³²T. Sugiyama, H. Kohara, M. Noda, and T. Nagasawa, "Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches," *Immunity* **25**, 977–988 (2006).
- ³³H. A. Himburg *et al.*, "Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche," *Cell Rep.* **2**, 964–975 (2012).
- ³⁴J. L. Liesveld, N. Sharma, and O. S. Aljntawi, "Stem cell homing: From physiology to therapeutics," *Stem Cells* **38**, 1241–1253 (2020).
- ³⁵A. Peled *et al.*, "Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4," *Science* **283**, 845–848 (1999).
- ³⁶C. H. Kim and H. E. Broxmeyer, "In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: Stromal cell-derived factor-1, steel factor, and the bone marrow environment," *Blood* **91**, 100–110 (1998).
- ³⁷S. Hatse *et al.*, "Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4," *FEBS Lett.* **527**, 255–262 (2002).
- ³⁸M. Z. Ratajczak, R. Reza, M. Wyszczynski, J. Yan, and J. Ratajczak, "Modulation of the SDF-1-CXCR4 axis by the third complement component (C3)—Implications for trafficking of CXCR4⁺ stem cells," *Exp. Hematol.* **34**, 986–995 (2006).
- ³⁹C. Scotti *et al.*, "Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering," *Proc. Natl. Acad. Sci.* **107**, 7251–7256 (2010).
- ⁴⁰C. Scotti *et al.*, "Engineering of a functional bone organ through endochondral ossification," *Proc. Natl. Acad. Sci.* **110**, 3997–4002 (2013).
- ⁴¹Y. S. Torisawa *et al.*, "Bone marrow-on-a-chip replicates hematopoietic niche physiology in vitro," *Nat. Methods* **11**, 663–669 (2014).
- ⁴²U. Blache *et al.*, "Dual role of mesenchymal stem cells allows for microvascularized bone tissue-like environments in PEG hydrogels," *Adv. Healthcare Mater.* **5**, 489–498 (2016).
- ⁴³S. Sieber *et al.*, "Bone marrow-on-a-chip: Long-term culture of human haematopoietic stem cells in a three-dimensional microfluidic environment," *J. Tissue Eng. Regen. Med.* **12**, 479–489 (2018).
- ⁴⁴K. Sayo, S. Aoki, and N. Kojima, "Fabrication of bone marrow-like tissue in vitro from dispersed-state bone marrow cells," *Regener. Ther.* **3**, 32–37 (2016).
- ⁴⁵M. C. Ciuffreda, G. Malpasso, P. Musarò, V. Turco, and G. Massimiliano, "Protocols for in vitro differentiation of human mesenchymal stem cells into osteogenic, chondrogenic and adipogenic lineages," *Methods Mol. Biol.* **1416**, 149–158 (2016).
- ⁴⁶J. Wu *et al.*, "The differentiation balance of bone marrow mesenchymal stem cells is crucial to hematopoiesis," *Stem Cells Int.* **2018**, 1540148.
- ⁴⁷M. J. Domingues, H. Cao, S. Y. Heazlewood, B. Cao, and S. K. Nilsson, "Niche extracellular matrix components and their influence on HSC," *J. Cell. Biochem.* **118**, 1984–1993 (2017).
- ⁴⁸M. V. J. Braham *et al.*, "Endosteal and perivascular subniches in a 3D bone marrow model for multiple myeloma," *Tissue Eng., Part C* **24**, 300–312 (2018).
- ⁴⁹N. Brandenburg and M. P. Lutolf, "In situ patterning of microfluidic networks in 3D cell-laden hydrogels," *Adv. Mater.* **28**, 7450–7456 (2016).
- ⁵⁰P. de la Puente *et al.*, "3D tissue-engineered bone marrow as a novel model to study pathophysiology and drug resistance in multiple myeloma," *Biomaterials* **73**, 70–84 (2015).
- ⁵¹M. V. J. Braham *et al.*, "Cellular immunotherapy on primary multiple myeloma expanded in a 3D bone marrow niche model," *Oncoimmunology* **7**, e1434465 (2018).
- ⁵²C. G. M. van Dijk *et al.*, "A new microfluidic model that allows monitoring of complex vascular structures and cell interactions in a 3D biological matrix," *Lab Chip* **21**, 1827–1844 (2020).
- ⁵³C. A. Saito-Reis, K. D. Marjon, E. M. Pascetti, M. Floren, and J. M. Gillette, "The tetraspanin CD82 regulates bone marrow homing and engraftment of hematopoietic stem and progenitor cells," *Mol. Biol. Cell* **29**, 2946–2958 (2018).
- ⁵⁴C. Y. Park, R. Majeti, and I. L. Weissman, "In vivo evaluation of human hematopoiesis through xenotransplantation of purified hematopoietic stem cells from umbilical cord blood," *Nat. Protoc.* **3**, 1932–1940 (2008).
- ⁵⁵P. Valent *et al.*, "Redistribution, homing and organ-invasion of neoplastic stem cells in myeloid neoplasms," *Semin. Cancer Biol.* **60**, 191–201 (2020).
- ⁵⁶S. Tavor *et al.*, "CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice," *Cancer Res.* **64**, 2817–2824 (2004).
- ⁵⁷L. Ding, T. L. Saunders, G. Enikolopov, and S. J. Morrison, "Endothelial and perivascular cells maintain haematopoietic stem cells," *Nature* **481**, 457–462 (2012).
- ⁵⁸F. Arai *et al.*, "Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche," *Cell* **118**, 149–161 (2004).
- ⁵⁹H. Yoshihara *et al.*, "Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche," *Cell Stem Cell* **1**, 685–697 (2007).
- ⁶⁰G. J. M. Maestroni, "Adrenergic modulation of hematopoiesis," *J. Neuroimmune Pharmacol.* **15**, 82–92 (2020).
- ⁶¹I. Coma *et al.*, "Process validation and screen reproducibility in high-throughput screening," *J. Biomol. Screen.* **14**, 66–76 (2009).
- ⁶²Y. González Hernández, "Serum-free culturing of mammalian cells—adaptation to and cryopreservation in fully defined media," *ALTEX* **24**, 110–116 (2017).
- ⁶³K. Pajtlér *et al.*, "Production of chick embryo extract for the cultivation of murine neural crest stem cells," *J. Vis. Exp.* **1**, 4–6 (2010).
- ⁶⁴D. L. Stemple and D. J. Anderson, "Isolation of a stem cell for neurons and glia from the mammalian neural crest," *Cell* **71**, 973–985 (1992).
- ⁶⁵I. Arganda-Carreras, R. Fernández-González, A. Muñoz-Barrutia, and C. Ortiz-De-Solorzano, "3D reconstruction of histological sections: Application to mammary gland tissue," *Microsc. Res. Tech.* **73**, 1019–1029 (2010).