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Tissue engineered models of healthy and malignant human bone marrow

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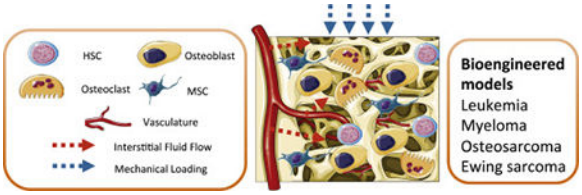
Abstract

Tissue engineering is becoming increasingly successful in providing *in vitro* models of human tissues that can be used for ex vivo recapitulation of functional tissues as well as predictive testing of drug efficacy and safety. From simple tissue models to microphysiological platforms comprising multiple tissue types connected by vascular perfusion, these “tissues on a chip” are emerging as a fast track application for tissue engineering, with great potential for modeling diseases and supporting the development of new drugs and therapeutic targets. We focus here on tissue engineering of the hematopoietic stem and progenitor cell compartment and the malignancies that develop in the human bone and bone marrow. Our overall goal is to demonstrate the utility and interconnectedness of improvements in bioengineering methods developed in one area of bone marrow studies for the remaining, seemingly disparate, bone marrow fields.

Graphical Abstract



Keywords

Human stem cells; Tissue engineering; Bone; Bone marrow; Bone tumors

1. Introduction

The bone marrow (BM) is a complex tissue with unique cellular microenvironments termed niches that support bone remodeling, hematopoiesis, and blood-mediated exchange of oxygen, nutrients and metabolites [1]. The organization of these cellular microenvironments, ranging from bone-proximal endosteal to the perivascular niches surrounding sinusoidal

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vessels, is critical for the proper function of the BM [2, 3]. Traditionally, *in vitro* cell culture and *in vivo* animal models have been used to investigate the homeostatic regulation of BM niches. These approaches however, have not been without limitations, including the lack of three-dimensional (3D) culture conditions and the reliance on animal tissues.

The recent shift in focus to 3D biomaterial platforms for BM modeling has allowed for spatial and temporal control of regulatory signals. Initially there were attempts to establish the scaffolding and culture conditions that were biomimetic to the required structural properties and necessary biosignals of the BM niches [4]. Interestingly, no single approach came to dominate the field and questions remained. Even following successes of tissue engineering in *de novo* recapitulation of the hematopoietic stem cell (HSC) niche, most models failed to demonstrate the hematopoietic diversity of native bone marrow, limiting their translational potential [2,5]. Still, these models have led to new ways to study and better understand the healthy human bone marrow [2]. In bone marrow malignancies, cell niches are disrupted and co-opted for pathogenesis.

Malignancies that begin in the bone marrow can be divided into: (*i*) *blood cancers* that develop from the blood-forming cells of the marrow [6–8], and (*ii*) *primary bone cancers*, commonly known as sarcomas, that form as solid tumors by the bone tissue cells themselves [9,10]. The most common hematological malignancies developing in the bone marrow are leukemias, followed by multiple myeloma [11], with a much lower incidence of bone marrow lymphoma [12]. The two most prevalent primary bone tumors are osteosarcoma and the Ewing's sarcoma family of tumors [13,14]. Osteosarcomas typically develop near the metaphyseal growth plate by cells believed to be malignant osteoblasts [15]. The Ewing's sarcoma family of tumors typically develop in osseous tissue [9] and are thought to originate from mesenchymal stem cells (MSCs) [16].

In *leukemia*, tissue-engineering approaches have centered largely on acute myeloid and lymphocytic leukemias (AML, ALL). Decades of traditional monolayer culture and *in vivo* animal models of AML and ALL have clarified many of the molecular determinants of leukemogenesis [17]. These models, unfortunately, have had reduced utility in elucidating the complex sets of cues that promote leukemia and induce cell resistance to chemotherapy [18,19]. Monolayer cultures require exogenous growth factors or stromal cells to maintain primary leukemic cell growth [17], and have been poor correlates to clinical outcomes [20–23]. Human leukemia xenografts, arguably the gold standard in leukemia modeling, have also had variable success as a pre-clinical model for drug screening [24]. In addition, xenografts necessitate the use of immunocompromised animals lacking the normal native bone marrow microenvironment that is critical for leukemic relapse [25]. Overall, up to 40% of primary patient samples fail to successfully engraft, further limiting the use of these models [26].

Multiple myeloma (MM) is a fatal B-cell malignancy involving destructive expansion of mutated plasma cells throughout the bone marrow, causing pathologies typically denoted as the mnemonic CRAB: elevated Calcium, Renal failure, Anemia, and Bone lesions [27]. Typically affecting those over 65, MM progression is closely tied to the disruption of the normal BM equilibrium between bone depositing osteoblasts and bone resorbing osteoclasts,

leading overall to greater osteoclastic activity and subsequent bone loss [28,29]. Known as the *vicious cycle*, MM induces a positive feedback loop process where increased osteoclast activity leads to release of growth factors from the extracellular matrix (ECM) that promote tumor growth, which, in turn, activates osteoclast-mediated bone breakdown, ultimately resulting in MM progression, bone lesions, and severe disruption of the normal bone marrow function. Because MM tumorigenesis and drug resistance are closely tied to its dynamic relationship with the bone microenvironment, current therapies target these interactions by using anti-resorptive agents like bisphosphonates [30–32]. The genetic heterogeneity of MM across patients (chromosal abberations, mutations) further complicates treatment [33]. Improved 3D models of MM within their human BM microenvironment are needed for the discovery of more efficient therapies.

Osteosarcoma (OS) is presented with a variety of distinct histological subtypes [34], with complex and genomically unstable karyotypes [35], and heterogeneity of the tumor matrix, vasculature, and immune cells [36]. The last 20 years of research have shown little improvement in patient survival rates, largely because of such extreme tumor heterogeneity and the lack of early detection markers. As a result, the cells of origin for OS and the early genetic events in OS have remained elusive, and the mechanisms controlling metastasis and relapse are poorly understood. Monolayer cell culture and mouse models have been used to unravel the mechanisms and pathways governing OS pathogenesis, with limited success. Cancer cell monolayers fail to recapitulate the complexity of the tumor BM microenvironment [37], while genetically engineered mouse models can be difficult and expensive to create, and are conceptually hard to design due to the inherent genetic heterogeneity of the OS [38]. Human orthotopic xenografts remain the gold standard for studies of OS, but they are not without weaknesses. In addition to the standard problems with *in vivo* mouse models (scalability, graft rejection, difficulty in studying pathways), intra-osseous OS injections are administered to immunocompromised mice lacking a normal hematopoietic BM microenvironment where OS forms [38,39]. An additional factor for modeling primary bone cancers is that unlike the hematological BM malignancies, OS involves the growth of a solid tumor with its own complex phenotype, structure, stromal interactions, and heterogeneity [40–42].

The *Ewing's sarcoma family of tumors (ESFT)* present a small round cell morphology, cell surface expression of CD99, and almost always feature a pathogenic chromosomal translocation of the *EWSR1* gene at the N terminus with an *ETS* gene at the C terminus [43]. The *EWS-FLI1* translocation is the most common, and can be observed in ~85% of all ESFT [44]. This is a rapid, aggressive, fatal malignancy believed to originate from BM-derived MSCs. Given the inaccessibility of targeting the nuclear EWS-FLI1 fusion protein, multimodal treatments have been developed mainly targeting the downstream activated genes and proteins as well as the tumor microenvironment that is heavily involved in ESFT tumorigenesis and metastasis [14,45–48]. While monolayer culture has been useful in large-scale drug screening of antineoplastic ESFT drugs, it has limited ability to identify drug candidates targeting the ESFT BM niche. Transgenic mouse models of ESFT have failed largely due to *EWS-FLI1* translocation related embryonic lethality, developmental defects, or failure to cause expression within specific target sites [49]. Orthotopic xenografts share similar challenges as those outlined for studying OS. Therefore, tissue engineering efforts

have focused on either trying to recreate ESFT tumor morphology and physiology, or on growing ESFT cells within ECM or scaffolds mimicking the BM niche. More recent models, including one pioneered by our group [50], aimed to create a more holistic model of ESFT.

This review discusses some new developments in the design and utilization of concepts and methodologies involved in the development of tissue-engineered 3D models of healthy and diseased BM, and their utilization in laboratory research and drug testing (Fig. 1).

2. Bioengineered models of human bone marrow

2.1 Cultures of hematopoietic stem/progenitor cells

Native human bone marrow is a complex, durable, and flexible mixture of inorganic bone mineral and ECM consisting of collagen fibers and proteoglycans [51]. Historically, a variety of synthetic and natural scaffolding biomaterials have been used to attempt to recreate the bone marrow (BM) niche, ranging from stiff porous analogs of trabecular bone to soft hydrogels [2, 52–56]. Interestingly, no single approach became the standard in the field, and instead several types of scaffolds were successfully used for expansion of hematopoietic stem and progenitor cells (HSPCs) *in vitro*, including poly(ε-caprolactone) (PCL), fibrin, and collagen [57]. Notably, human cord blood mononuclear cells were used to create bone marrow *in vitro*, without the need for addition of cytokines [58].

By coating the biocompatible, highly porous polyurethane scaffolds with collagen type 1, the cells were provided with endosteal-like mechanical stiffness and porosity, cell adhesion sites, and interactions promoting their growth. Furthermore, this approach demonstrates that a combinational method of incorporating a scaffold mimicking the architecture of the spongy bone marrow coated with an ECM protein native to bone like collagen type 1 is a successful strategy for improving the *in vitro* expansion of the hematopoietic compartment of bone marrow. Nonetheless, there is still a great deal of bone mineral and ECM complexity missing from these engineered models, the components that play crucial roles in the maintenance of the healthy BM niche. For example, the approximation to native mineralized bone for healthy bone marrow tissue engineering involves the incorporation of hydroxyapatite into the scaffolds, while still missing the structural organization and composition of minerals in the bone [51, 59].

The human BM niche also features an incredible diversity of cell types, namely osteoblasts, osteoclasts, vascular cells, hematopoietic stem and progenitor cells, and mesenchymal stem cells among others. Given the role of mesenchymal stem cells as stromal cells within the BM niche supporting CD34+ HSPC maintenance [60,61], most approaches for now rely on co-culture with MSCs [57,62–64]. For example, Jing et al explored the effect of the spatial relationship between HSPCs and MSCs on *ex vivo* HSPC expansion and phenotype [65]. Two primary HSPC compartments were found in co-cultures of HSPCs and MSCs: one at the MSC stromal surface that fosters HSPC proliferation, and the other below the MSCs that maintained the HSPCs in a more quiescent, immature state. Using a collagen gel matrix, Leisten et al recapitulated the two HSC microenvironments [66], each with a distinct effect on HSC fate (Fig. 2A). HSPCs in suspension on collagen gel became highly proliferative and differentiated into maturing myeloid cells (CD38+, CD13+, CAE+) and natural killer

cells (CD56+). The HSPCs that migrated into the collagen gel seeded with stromal MSCs maintained a more primitive CD34+/CD38- phenotype. This study showed that the source of MSCs plays an important role in maintaining HSPCs and MSCs, with bone marrow derived MSCs outperforming umbilical cord derived MSCs that often resulted in the loss of HSPC lineage commitment and differentiation.

It is becoming clear that not only is the inclusion of MSCs in culture with HSPCs necessary for maintenance of the long term quiescent phenotype, but the source of the MSCs is important as well. Umbilical cord blood, which is abundant, widespread, and rich in HSCs, meanwhile appears to be a preferred source of HSCs [2,67]. Missing from these co-cultures are the numerous other cell types native to the BM niche, which likely also interact with HSCs and mediate their phenotype. Osteoclasts, the cells responsible for bone resorption, for example are known to play important roles in HSC regulation and mobilization [68].

Other groups have implemented further modifications to the culture conditions, by altering the oxygen concentrations [69] and introducing fluid flow [70], to mimic hypoxia and mechanical stimuli experienced in the native BM niche respectively. Reduction of oxygen concentration to 10% (one half of normoxic) promoted expansion of bone marrow mononuclear cells in culture [71]. Cultivation of MSCs in hydrogels created a hypoxic microenvironment beneficial for HSPC support, while subsequent re-oxygenation eliminated these advantages in maintaining yields of the desired $CD45^{+}34^{+}38^{-}Lin^{-}$ primitive HSPCs [64]. Perfusion associated with shear stresses has also been introduced into BM modeling [72]. For example, perfusion culture of hematopoietic progenitors in hydroxyapatite scaffolds improved the colony formation capabilities of HSPCs over 6 weeks in hollow fiber bioreactors [73]. These promising results show that the current models could benefit from fine-tuning of both oxygen levels and interstitial flows throughout the scaffolds to better mimic the native BM niche replete with HSPCs.

2.2 Bone marrow on a chip

Despite successes in the recapitulation of the HSC niche, the existing models have failed to demonstrate the hematopoietic diversity observed in native bone marrow, limiting the translational potential of this approach. Generation of a mouse bone marrow-on-a-chip was the first tissue engineered model featuring *in vivo*-like proportions of hematopoietic stem and progenitor cells that could be autonomously maintained for a minimum of 1 week [74]. This approach involved a microfabricated polydimethylsiloxane (PDMS) device with a cylindrical cavity filled with collagen gel, demineralized bone powder, and bone morphogenetic proteins that was implanted subcutaneously into mice for 8 weeks and returned into the microfluidic platform. This multi-step *in vivo/in vitro* approach allowed recapitulation of the complex structural, physical and cellular microenvironment of whole bone marrow, and modeling of marrow radiation toxicity responses and effects of protective drugs in perfusion cultures.

In this approach, a mouse host is required to repopulate the perfusable device with all of the marrow cellular components, such that the model can only feature mouse bone marrow, which is well known for its limitations in representing native human marrow [75]. Building on the knowledge generated from human tissue engineered BM models, a recent effort

resulted in the first xeno-free, human bone marrow-on-a-chip (Fig. 2B) [59], established in a co-culture of BM-derived MSCs and cord blood derived HSPCs in hydroxyapatite-coated zirconium oxide ceramic scaffolds. In a microfluidic multi-organ platform, the HSPC cultures could be maintained for at least 28 days in a primitive CD34+ CD38- state capable of granulocyte, erythrocyte, macrophage, and megakaryocyte differentiation. The MSCs deposited fibronectin ECM and expressed stem cell factor (SCF), nestin, and osteopontin (OPN), all of which are known to aid to long-term maintenance of HSPCs [59,76–78]. This human cell based model can be scaled up, with the potential to act as an *ex vivo* CD34+ HSPC expansion system. There is also a possibility for vascularization, since the hydroxyapatite scaffold can promote the formation of microvascular networks [79].

Bourgine et al went even further, showing that co-culture of HSPCs with MSCs and osteoblasts in a hydroxyapatite-coated ceramic scaffold under perfusion allows for compartmentalized recapitulation of the biphasic character and behavior of HSPCs in the BM. To this end, naïve $CD34^{+}$ $CD38^{-}$ HSPCs were maintained within the scaffold stroma while HSPCs committed to differentiation like granulocyte-monocyte progenitors were actively being secreted into the circulating “blood” (Fig. 2C) [80]. Furthermore, some of the compositional and structural features of native BM (namely secretion of a complex mixture of collagen type 1, collagen type 4, fibronectin, and osteocalcin ECM) were recreated. Finally, the two phenotypes of HSPCs were maintained in serum free media with reduced concentrations of cytokines as compared to prior culture techniques. The remarkable progress made here demonstrates the need for sophisticated multi-dimensional modelling that has arguably been hindered by the lack of inter-disciplinary approaches to tissue engineering healthy and malignant BM niches.

2.3 Modeling of human bone marrow niche interactions

There has also been a variety of tissue engineering approaches employed towards the elucidation of HSC biochemical and biophysical interactions with their surrounding BM niche. For example, the *in vivo*-like human bone marrow on a chip model proposed by Sieber et al [59] served as a tool for studying human BM niche specific interactions. In collagen hydrogels blended with polyacrylamide (PA) substrates with tunable elasticity, HSPC morphology was significantly impacted by biophysical cues, and the cell viability was maximized at the 0.044 kPa substrate stiffness and 100 μg/mL collagen hydrogel concentration [81].

In a seminal effort to mimic the functional hematopoietic stem cell niche *in vitro*, Sharma et al showed the power of 3D tissue engineering methodologies for dissecting the roles of individual cell populations in the BM [64]. Prior *in vitro* and *in vivo* mouse models suggested a role for osteoblasts in HSC quiescence and HSC-supportive properties of MSCs [64,82,83]. Moreover, while it was well known that the stromal cell-derived factor 1 alpha (SDF1α) / C-X-C chemokine receptor type 4 (CXCR-4) axis was critical to retention of HSCs in the BM [84], this model demonstrated that the MSCs specifically are responsible for enriching the environment with the necessary SDF1α chemokine. Chemical disruption of this axis by addition of the immunostimulant AMD3100 resulted in egress of the HSCs from the matrix as seen *in vivo* [85], suggesting a novel role of such systems for screening of

HSC-mobilizing agents. This simple hydrogel scaffold design also resolved some of the controversy from *in vivo* studies about the role of N-cadherin in HSC function [86], by showing that a large proportion of the CD34+ HSPCs stained positive for N-cadherin, unlike in monolayer cultures. This model also offered new insights into the regulatory pathways in the HSC niche. It was shown that 3D-MSCs express HSPC-supportive transcriptomes and proteomes when compared to MSC monolayers: a lack of CD146 and significantly higher expression of osteopontin (OPN), runt-related transcription factor 2 (Runx2), angiopoietin, nestin, and integrins α4 and β1 (VLA-4). Using this model, cell specific regulation of the HSC niche allowed novel insights into the BM niche and a powerful tool for the dissection of HSPC communication with other resident BM cell types such as endothelial cells.

Tissue engineered modeling of the BM niche also allows modulation of the ECM, creating a novel and effective tool for investigating the biochemical cues in the 3D BM microenvironment. As an example, one significant limitation of *in vivo* models is that these cues are available to HSCs in both the soluble and insoluble form, subsequently confounding results [87,88]. In order to resolve this issue, tissue engineers have used acrylate-functionalized polyethylene glycol (PEG) to tether and immobilize SCF within methacrylamide-functionalized gelatin (GelMA) hydrogels [89]. By doing this, it was shown that the insoluble SCF form promoted the maintenance of primitive HSCs while the soluble form induced proliferation of differentiating HSPCs [89]. This same group also creatively exploited the tunable nature of these 3D models to explore the thresholds of autocrine and paracrine cues and their ability to alter HSC fate decisions [90]. Hydrogel diffusivity and cell density in the niche were co-varied, with the combination of high diffusivity and high Lin+ cell density lending to paracrine dominated signaling, while the other end of the range resulted in domination of autocrine factors. Within the autocrine microenvironment, the HSPCs were insulated from Lin+ cell signaling, and became more enhanced towards myeloid lineage specification as the system became friendlier to paracrine signaling.

A more recent study resulted in a 3D model of BM that incorporated both the biochemical and biophysical cues involved in stem cell fate decisions [91–93]. A modular star-shaped PEG (starPEG) - heparin hydrogel system was further used to recapitulate the glycosaminoglycan (GAG) rich BM with GAG-mediated growth factor presentation (Fig. 2D) [91]. In this model, the increased spatial confinement and GAG-rich microenvironment increased the viability and therefore frequency of quiescent HSCs, with functionality of these cells observed at similar levels compared to non-cultured HSCs. While it is clear how these technologies will be used in the future to further clarify the relationships of HSCs with their surrounding ECM, they could also easily be applied towards future studies into the interactions of the various malignancies of the BM with this same niche.

2.4 Translational capabilities of 3D bone marrow models

In addition to being invaluable for investigating the dynamics of the human BM niche, tissue engineered models also have immense clinically translational potential. The mouse bone marrow-on-a-chip already offered a tool for determining drug responses, radiation toxicity, and reparative efficacies of bone marrow constituent cells [74]. The human bone marrow-on-a-chip could only assess these parameters on undifferentiated HSPCs [59], while also

serving as an *ex vivo* CD34+ HSPC expansion system for applications in regenerative medicine and modeling of chemotherapeutic dosing. Bourgine et al already showed that their perfusion HSPC system could be used to study injury responses ex vivo by applying bleomycin, and observing reduced capacity for the stromal MSCs to maintain the quiescent HSC population [80]. Notably, these models employ microfluidics enabling the analysis of pharmacokinetic and pharmacodynamic drug behaviors under flow conditions [59,74].

A perfused bone marrow analog was shown to be capable of mimicking the HSC quiescence needed for long term culture, as well as an "activated" condition stimulating HSC differentiation, which made the model amenable to drug testing [94]. In HSPC/MSC co-cultures in arginylglycylaspartic acid (RGD)-peptide coated macroporous PEG hydrogel resembling trabecular bone, accumulation of cytokines, hypoxia, and a higher frequency of CD34+ HSPCs were observed within the first 9 days in static cultures relative to perfusion cultures. In contrast, hydrogels in perfusion demonstrated hematopoietic differentiation, suggesting that perfusion can be used to activate the switch from the dormant to the proliferative and differentiating state of the HSC. Additionally, in proof-of-concept experiments with the myelotoxic chemotherapeutic 5-fluorouracil, the toxicity was strongly underestimated in monolayer cultures, while the 3D conditions had a significant impact on the way hematopoietic cell subpopulations reacted to the drug, indicating the application of this modular model as a testing system for drug toxicity on either the quiescent or proliferative HSCs.

Other groups have taken an entirely different approach, by utilizing tissue engineering for translational applications that are unrelated to HSCs, but still pertain to bone marrow. Megakaryocytes that are derived from HSPCs and matured in bone marrow are responsible for forming and releasing platelets necessary for blood clotting in response to injury [95–98]. Excessive bleeding, sepsis, cancer-related thrombocytopenia, among other medical indications, can all necessitate administration of platelet transfusions [99]. In an effort to address this need for *ex vivo* produced platelets, a modular flow chamber was established featuring a silk fibroin scaffold and enabling efficient formation of platelets and release of megakaryocytes and recoverable platelets [100]. This model was later expanded into a perfusion bioreactor system with tight control of scaffold architecture and flow behavior [101]. This approach could potentially be utilized to study HSCs in the BM towards expanding the capabilities of patient- specific HSPC expansion ex vivo, which is an urgent clinical need.

2.5 Incorporation of vasculature in 3D bone marrow models

While an in depth look at microvascular models is outside the scope of this review, we will discuss some notable attempts to implement these models to tissue engineered BM. Co-culture of HSPCs with vasculature is a desirable yet largely unachieved goal, based on the critical role of endothelial and perivascular cells to maintain HSCs in vivo [18]. The majority of approaches to vascularization of tissue-engineered bone has been motivated by the need to improve surgical integration of bone grafts [102]. For two decades, our lab has been focused on innovations in bone tissue engineering, primarily for the generation of anatomically shaped human bone grafts [103–105]. One approach involved encapsulation of

human umbilical vein endothelial cells (HUVECs) and MSCs within a fibrin gel, and subsequent culture of these cells in decellularized trabecular bone scaffolds [106]. In these studies, we made two observations: (*i*) vascular development had to be induced prior to osteogenesis; and (*ii*) additional MSCs needed to be added at the osteogenic induction stage to improve osteogenesis and vascular networking. In another study, osteogenic human bone marrow derived MSCs were cultured in collagen gels adjacent to a channel coated with endothelial cells [107]. Breast cancer cells were subsequently injected into the endothelial channel and allowed to transmigrate into the bone environment. Subsequently, functional microvascularized bone was established by using a triculture consisting of endothelial cells, osteoblast-differentiated cells, and MSCs [108].

All of these models however rely on the supplementation of angiogenic growth factors that could negatively impact or confound quiescent HSC culture and were deprived of biomechanical stimuli [106,107]. Most recently, our group created a novel human bone perivascular niche-on-a-chip and bioreactor that largely overcame these obstacles and exposed the niche to biomimetic interstitial fluid flows (Fig. 3A) [109]. We showed the feasibility of recapitulating a perfused bone perivascular niche-on-a-chip to study the progression of breast cancer colonizing the bone and the drug resistance of metastasized cells.

The niche-on-a-chip enabled control of vascular flow (Fig. 3A), shear stress (Fig. 3B) and oxygen gradients (Fig. 3C), resulting in a robust and stable vascular network, at ~10 fold reduction in the concentration of exogenous angiogenic factors. Human bone marrow-derived MSCs underwent transition toward perivascular cell lineages, and supported the formation of capillary-like structures lining the vascular lumen. Most importantly, breast cancer cells in this environment maintained the slow-proliferative state associated with aggressive metastasis and drug resistance. We were able to generate stable, long lasting, extensive capillary-like networks that self-assembled, within the 3D native bone matrix. The cultivation on microfluidic chip that allowed tight control of flow velocities, shear stresses, and oxygen gradients. Moreover, we were able to show that fluid flow was specifically responsible for the promotion of a slow-proliferative state of metastasized breast cancer cells that increased their drug resistance. Clearly, this type of tightly controlled perfusion model could also be applied towards studying HSCs in a healthy bone marrow niche and various BM malignancies, in response to physiologically relevant biophysical and biochemical stimuli.

3. Modeling acute leukemia in the engineered bone marrow microenvironment

3.1 Modeling acute leukemia ex vivo

Major efforts have been made to optimize the biomimetic BM environment for growing leukemia cells. Cell lines for leukemia subtypes have been successfully cultured in porous scaffolds generated from biodegradable and non-biodegradable materials [110], such as poly (L-lactic-co-glycolic acid) (PLGA) and polyurethane. Biorecognition signals have been included in the form of ECM proteins, collagen 1, or fibronectin, with collagen 1 yielding

the most sustainable (8 weeks) leukemic cell culture without the addition of exogenous factors [110]. Decellularized Wharton's jelly, the gelatinous material of the umbilical cord, has also been used as a matrix for leukemic stem cell (LSC) culture [111], acting as a functional substitute for BM ECM as it is composed of similar components (collagen, fibronectin, and hyaluronic acid with its CD44 receptor) [111–113].

While other attempts at modeling leukemia relied on achieving cell proliferation *ex vivo*, Li et al attempted to recapitulate specifically the LSC niche [114], given that this quiescent population has been implicated in leukemia relapse [115–117]. Leukemic cell lines were cultured without differentiation within decellularized Wharton's jelly matrix, with decreased proliferation, prolonged dormancy, a spindle-shaped morphology, and increased expression of the CD44 cell adhesion surface marker that is suggestive of an LSC phenotype. Deeper characterization of the cellular profiles suggested that the enrichment of LSCs rather than other progenitors was causing increases in these markers of self-renewal ability [114].

Identification of the ECM scaffold for leukemia modeling is, however, only one component of the BM model. As with modeling of healthy bone marrow, it is important to include additional microenvironmental factors, such as shear stress [118] and MSCs [119] that play a yet-unclear role in leukemia. A critical effort by Houshmand et al attempted to consolidate all of these elements of the leukemic BM niche by generating the first microfluidic platform with a bone scaffold for mimicking the AML BM niche [120]. Demineralized bone matrix, capable of secreting critical BM growth factors like bone morphogenetic protein 2 (BMP-2), transforming growth factor beta 1 (TGF-β1), and vascular endothelial growth factor (VEGF) [121], was coated with collagen type I, and used to co-culture MSCs and erythroleukemic TF-1 cells subjected to slow interstitial flows within a microfluidic device [120]. Under these conditions, leukemic cells showed retention of CD34/CD38/CD33 expression, a remarkably higher proliferation rate, and increased drug resistance relative to 2D culture. Of note, leukemic cobblestone-like clusters [122], that are associated with the maintenance of leukemia, were observed [120].

Attempts at modeling acute ALL, the malignant transformation and proliferation of lymphoid progenitor cells in the bone marrow and other sites, have also been made. Unlike AML, which largely targets adults, ALL is a disease targeting children [123,124]. In 2015, the first 3D microfluidic model of ALL was reported [126], comprised of a viable tri-culture of bone marrow-derived MSCs, osteoblasts, and SUP-B15 ALL cells in PDMS matrices subjected to perfusion-derived fluidic shear stresses. The stromal cells have since been shown to play key roles in ALL maintenance *ex vivo*. Co-culture of patient-derived ALL samples with nestin-positive MSCs allowed for the long-term (>8 weeks) maintenance of clonal abundance and self-renewal potential [126].

Finally, patient specific *ex vivo* models of leukemia have also been developed. One such study involved a high-throughput platform where patient derived AMLs were screened for viability against exposure to over 200 U.S. FDA-approved chemotherapeutics across a 10,000- fold concentration range [127]. Predictive simulation allowed collection of individualized genomic data for deregulated pathways and identification of candidate drugs. In a separate study, primary bone marrow mononuclear and malignant cells were isolated

from patients with a variety of hematological malignancies like leukemia, lymphoma, and multiple myeloma and maintained for over 30 days in a reconstructed bone ECM matrix hydrogel scaffold consisting of Matrigel, fibronectin, and collagen type 1 [128].

Overall, there have been substantial advances in leukemia tissue engineering, allowing for the prolonged and successful culture of both cell lines and patient materials in 3D settings mimicking the BM niche. The challenge remains to build on the complexity of such models by, for example, incorporating vascular cells, similar to how they should be added to models of healthy BM. It is already established that AML and ALL cell viability, cycling, and resistance to chemotherapeutics are regulated by the vascular niche [129,130]. There are already methods for engineering a vascularized BM niche, as has been demonstrated in this review, that could be applied to future 3D *in vitro* modeling of AML and ALL.

3.2 Leukemia-bone marrow interactions and drug resistance

In vitro engineered models have been particularly useful in clarifying the roles of cell-cell interactions in leukemia resistance. Co-culture of human BM-derived MSCs and leukemia cell lines (HL-60, Kasumi-1, and MV411) in a synthetic polyglycolic acid/poly L-lactic acid (PGA/PLLA) scaffold revealed higher resistance to therapy-induced apoptosis in this 3D model as compared to cell monolayers, with cell-specific responses [131]. Perhaps most interestingly, N-cadherin was significantly upregulated in cell lines that showed greater resistance in the 3D model, suggesting a novel role of this cell adhesion protein in leukemia resistance.

Another approach involved the use of BM-derived MSCs from patients with AML and ALL to create cell spheroids that were then exposed to leukemia cells [132]. The spheroids demonstrated layer-dependent and patient-specific physiological differences: less reactive oxygen species at the center of the spheroids indicative of hypoxia (as in the BM leukemia niche), along with decreased expression of the chemokine SDF1α. Critically, it was observed that AML cells homed to the centers of the stromal spheroids and exhibited resistance to cytarabine (a chemotherapeutic) as a function of the location within the spheroid.

In separate studies, a tri-culture of MSCs, endothelial cells, and leukemia cell lines in matrix metallaproteinase-sensitive hydrogels mimicking the perivascular niche showed greater resistance to daunorobucin and cytarabine than the corresponding cell monolayers [133]. This finding aligns with the observations that endothelial cells improve proliferation of AML cells [134], and that patients with hematologic malignancies exhibit higher microvascular density in their BM [135], which may be tied to drug resistance [136,137]. The addition of the CXCR-4 inhibitor, AMD3100, a first line of defense therapy that is believed to target the BM microenvironment, mobilized the AML cells away from the vascular cells [85], showed the utility of this model and the importance of co-culturing leukemia cells in their native human niche. These studies demonstrate the need for incorporating more complexity, in particular endothelial cells, into tissue engineered models of leukemia to better mimic the malignant BM niche, a recurring theme in this review.

Cell-matrix interactions were also shown to promote drug resistance in 3D cell models. Culture of AML cells within a fibronectin-coated polyurethane/poly-L-lactic acid (PLLA) micronanofibrous scaffold mimicking the architecture of decellularized bone marrow allowed the maintenance of the $CD34^{+}/CD38^{-}/CD33^{-}$ AML cell phenotype and displayed high cell adhesion, in addition to high adhesion-mediated resistance to cytarabine and daunorubicin [138]. Moreover, the B-cell lymphoma 2– cyclin-dependent kinase inhibitor 1B (Bcl2-p27Kip1) pathway of cell cycle control associated with chemoresistance *in vivo* was also observed. Abrogation of this pathway using the Bcl-2-specific inhibitor ABT 737 eliminated chemo-resistant advantages.

It is still unclear how ECM stiffness impacts leukemia growth and drug efficacy *in vivo*. Cultures of AML cell lines in mechanically tunable alginate hydrogels with a broad spectrum of stiffnesses and RGD-integrin ligand densities showed cell line specific effects of matrix stiffening and ligand density on proliferation and chemosensitivity. Implantation of these devices into *in vivo* xenografts corroborated these findings, suggesting that stiffness acts as a dominant factor in tumor growth and drug response (Fig. 4A) [139]. Overall, these studies contribute to other BM tissue engineering fields, as they provide an easily reproducible 3D scaffolding system with easily tunable stiffness and ligand binding, that cab mimic the broad range of biophysical parameters across the BM niche.

4. Modeling multiple myeloma (MM) in the 3D BM niche

4.1 Patient-derived models of multiple myeloma

The need to incorporate the BM microenvironment that provides tumor progression signaling and chemo-resistance in 3D models of MM has been evident for many years. In the first tissue engineering breakthrough a decade ago, Kirshner et al presented a Matrigel-based culture that enabled the first *ex vivo* clonal expansion of MM plasma cells [140], and used this model to test anti-tumor drug specificity targeting particular clonal compartments.

Unlike approaches used to model other BM malignancies, the cultures of normal and diseased tissues were the most relevant way to study MM progression in the human context. This approach stands in stark contrast to the other BM niche tissue engineering fields that are in many ways still heavily dependent on cell lines and commercially available primary cells, limiting their potential towards studying the effects of patient heterogeneity on the BM niche. Applying methods developed for *in vitro* culture of rat bone explants [141], Ferrarini et al maintained long-term dynamic cultures of excised MM patient tissues in a Rotary Cell Culture Bioreactor (RCCS™) [142], and demonstrated the viability of the myeloma cells and the presence of key components of the BM microenvironment. This system was used to evaluate chemotherapeutic effects within the context of a complex tissue: the proteasome inhibitor bortezomib widely used to treat MM negatively impacted both the MM cells and the blood vessels. Notably, the effects of drugs measured in this system were similar to those measured in patients. Another approach was developed to grow the patient derived MM cells within an engineered ossified tissue substrate supplemented with patient-derived plasma, in a perfused microfluidic device [143]. Here it was observed that the osteoblast-generated endosteal-like layer was critical for *ex vivo* preservation of primary MM cells.

Despite their ability to accurately mimic BM physiological conditions, these patient tissue-based systems have some major drawbacks. Tissue explants grown in the RCCS™ bioreactor were difficult to scale, and there were issues with reproducibility and tissue harvesting. The MM cells grown in engineered ossified tissue heavily depended on the presence of healthy osteoblasts, which causes reproducibility issues due to the differential effects of these cells on MM progression and the stromal tissue [144, 145]. A novel tri-culture system was established for a 3D tissue engineered bone marrow (3DTEBM) using matched primary MM cells and their malignant microenvironment, with drug gradients and hypoxia, and limited addition of exogenous factors [146]. This system allowed modeling of human MM in a patient specific and technically facile fashion (Fig. 4B).

Most recently, this model was shown to be a much better predictor of clinical efficacy of drugs than the 2D *in vitro* cultures [147]. A literature search revealed that there was no correlation (correlation coefficient of R2 = 0.007) between IC50 values determined *in vitro* and efficacious clinical concentrations, while the 3DTEBM's experimental IC50 values showed a clearly contrasting direct correlation (correlation coefficient of R2 = 0.979). These highly impressive drug efficacy findings obtained using the 3DTEBM technology suggest that this is a highly promising approach for studying primary BM malignancies, and in particular those of hematologic origin such as AML and ALL.

4.2 Recreating multiple myeloma in a bioengineered bone marrow niche

The patient-derived biologically relevant MM models share limited controllability, scalability, adaptability, and reproducibility [148]. For these reasons, 3D tissue engineered models of MM have been developed using a bottom-up approach. Such models have proven useful for investigating MM biology and potential new therapies. A porous silk scaffold for active cell attachment was used to identify a novel microRNA signature (miR-199a) associated with cell adhesion-mediated drug resistance and the target microRNAs involved in the reactivation of osteogenesis in MM-afflicted MSCs [148]. In another study, functional MM tissues were grown from BM-MSCs, HUVECs and primary MM cells in gelatin scaffolds, and maintained in RCCS™ bioreactors [149]. The vascular cell adhesion molecule 1 (VCAM1) transfectants were used to activate the VLA-4 - VCAM1 pathway to bortezomib resistance, a novel and promising therapeutic developed against MM.

A bioprinting method has been used to create separate endosteal and perivascular subniches of the MM-BM, and demonstrate the essential role of the perivascular niche in supporting MM [150]. Such an approach could prove particularly useful across studies into healthy and malignant bone, given the well noted effects both niches have on maintaining HSCs, leukemias, and primary bone tumors. Novel therapies and biological cues and drug resistant pathways have also been explored using such models, such as the application of cellular immunotherapy to MM within a 3D BM niche model [151]. αβ-T cells engineered to express a tumor-specific γδ T cell receptor displayed infiltrative homing to the MM cells. The therapeutic potential of immunotherapies is immense, and methods used here could also be applied in tissue engineering of other BM malignancies.

5. Modeling Ewing Sarcoma in the 3D BM Microenvironment

5.1 Generating Ewing sarcoma tumors in a 3D bone marrow niche

The methods involved in modeling ESFTs have been quite similar to those used in studying osteosarcoma, which is unsurprising given that both are solid tumors arising from bone tissues often within the bone marrow niche. Historically, the focus has been on either attempting to mimic the solid tumor's structure and therefore biological phenotype, or to grow the cancer cells within a scaffold mimicking the ECM and trabecular bone architecture, allowing for modeling of cell-ECM adhesion, growth, and migration [152,153]. Spontaneous multicellular tumor aggregation, which generates tumor-like spheroids, can be achieved by inhibiting cell adhesion to solid surfaces and thereby promoting cell-cell adhesion. ESFT 3D spheroids closely mimic the growth rates, cell-cell junctions, kinase activation, and cell morphology of primary Ewing's sarcoma (ES) tumors [154]. Remarkably, the self-assembled tumor aggregates feature pH, nutrient, and hypoxic gradients across distinct zones: a central necrotic core, a quiescent middle layer, and a proliferative outer layer [152,153,155]. The similarity of ESFT spheroids to *in vivo* tumors have made them useful tools in the investigation of cell signaling pathways and identification of cell surface markers with potential for immunotherapeutic strategies [152, 156–162].

Tissue engineered scaffold approaches have been used to recapitulate the complex BM tumor microenvironment of ESFT that provides critical architectural, mechanical, and biochemical cues for tumor expansion and metastasis. Collagen supplemented hydrogel matrices and fibrous scaffolds, particularly made from PCL, polylactide (PLA), PGA, and co-polymers (PLGA), have been useful for growing ES cancer cells in 3D, investigating their interactions with the ECM, and cell-adhesion mediated drug resistance [152]. Surprisingly, most of the scaffolding approaches have not incorporated other cell types residing in the BM niche. For example, vasculogenesis driven by native BM cells is essential for ES tumorigenesis [163]. One early effort used a co-culture of ESFT and endothelial cells in Matrigel™ to show formation of vascularized endothelial tubes [164].

Our group established a bioengineered model of ES within the native bone tumor niche [50], by creating ES tumor aggregates in culture, seeding them into a mineralized trabecular bone scaffold, and co-culturing them with human osteoblasts. This was a completely novel approach in which we combined both prevailing methodologies for studying ES in 3D: generation of tumor spheroids and culture of ES cells within a BM-like matrix. With this model, we were able to recapitulate the hypoxic, glycolytic tumor phenotype with a necrotic core surrounded by proliferative ES cells, consequently enabling re-expression of transcriptional profiles related to focal adhesion, malignant deregulation, angiogenesis, and vasculogenic mimicry as observed in patient tumor samples. Additionally, we were able to identify two new candidates for ES targeted therapy: *CDC42* and *PPP1R12A*. This example demonstrates the improvements in biological fidelity of BM models, towards more closely resembling the native healthy and malignant BM niche.

Osteoclasts play a major role in the growth and migration of malignancies in the BM, therefore we incorporated functional human osteoclasts into this bioengineered model of ES

(Fig. 5A) [165]. The mix of functional osteoblasts and osteoclasts expectedly led to bone matrix deposition and resorption resulting in calcium release and bone remodeling as would be seen in the native BM niche. Co-culture of this model with ES tumor spheroids, known to activate bone-resorbing osteoclasts, predictably led to a reduction in bone density, connectivity, and matrix deposition. We also showed that this “vicious cycle” could be prevented with the application of zoledronic acid, a bisphosphonate therapeutic agent typically used to treat osteoclast-mediated bone loss [166]. Given the importance of osteoclasts in healthy homeostasis and disease pathogenesis of the BM, it becomes clear how the technology developed using this osteolytic ES bone model could be applied in other types of healthy and malignant BM studies, and perhaps towards other bone diseases not elaborated upon such as osteoporosis.

In recent years there has been increased interest in the role of exosomes in cancer modulation of the microenvironment [167–169]. Exosomes are small membrane vesicles containing bioactive molecular cargo that are released by cells and may have cell specific targeting [170]. In the first study of its kind, we demonstrated that we could generate exosomes, using our bioengineered ES model, that recapitulate some of the properties of exosomes released by native human ES tumors, notably their size distribution, high mRNA levels of the chromatin modifier polycomb histone methyltransferase EZH2, and targeting of surrounding MSCs (Fig. 5B) [171].

5.2 Evaluating the effects of biophysical stimuli on Ewing sarcoma culture

In addition to the studies of ES tumor spheroids and 3D BM-like scaffolds, there have also been investigations into the role of native BM-like biophysical stimuli such as fluid flow and their effects on ESFT. BM stromal osteoblasts grown in 3D culture have been shown to exhibit higher amounts of mineralized matrix deposition with increasing fluid shear forces [172]. Expanding on this, Santoro et al decided to introduce flow perfusion into their electrospun PCL 3D scaffold model of ES [173]. Static culture alone in PCL scaffolds showed a more native like ES cell phenotype [174] and significant upregulation of the insulin-like growth factor-1 receptor (IGF-1R) pathway than that observed in classic monolayers [175]. Exposure of this ES model to flow-derived shear stress further improved the culture outcomes, activated mechanotransduction pathways, and resulted in strikingly higher insulin growth factor 1 (IGF-1) ligand production and secretion that could competitively outcompete IGF-1R inhibitors, thus increasing resistance to that class of therapeutics [173].

In a later effort, Santoro et al introduced MSCs into perfused ES scaffold bioreactors to demonstrate how ES cells can acquire drug resistance by co-opting neighboring stromal cells of the BM niche, something observed in patients that can become convoluted during *in vivo* investigations [176]. Co-culture of ES cells with MSCs leads to significant increases in interleukin 6 secretion, which causes signal transducer and activator of transcription 3 pathway activation in the MSCs and results in dysregulation of the IGF-1 pathway, skewing towards secretion of even more IGF-1 ligand that can outcompete IGF-1R inhibitors. Given some of the structural and microenvironmental similarities between ES and OS and that IGF-1R inhibitors are also being looked into as therapeutic agents against OS, it may prove

beneficial to try to use the tissue engineering methodologies described here towards modeling OS response and potential resistance to this class of drugs [177].

Recently, another effort used 3D printing combined with flow perfusion bioreactors to create scaffolds with inverse porosity and shear stress gradients in order to better understand the gradient dependent mechanotransductive regulation of the IGF-1 pathway that plays a major role in ESFT pathogenesis [178]. Our group was the first to investigate the effects of mechanical loading on ES cells [179], in co-cultures of ES cells and MSCs in a collagen type 1 matrix (Fig. 5C) [180]. We restored the extracellular signal–regulated kinases 1/2 (Erk1/2) - Runx2 signaling pathway seen in patients by mimicking BM-niche like mechanical stimuli. Importantly, the rescue of Runx2 activity by this mechanobiological mechanism resulted in increased drug resistance to receptor tyrosine kinase inhibitors like sorafenib, imatinib, and sunitinib that showed promise in preclinical studies, but ultimately failed in ES patients. Also, this is another methodology developed in one field of BM tissue engineering that could easily be applied to other fields in order to determine, for example, the effects of mechanical stimuli on HSPC homeostasis or leukemia drug resistance.

6. Modeling osteosarcoma in the 3D BM environment

6.1 Generating osteosarcoma tumors in a 3D bone marrow niche

There have been two main approaches to 3D OS culture that bear striking resemblance to the methods previously described for modeling the ESFT: (*i*) scaffold-free spontaneous aggregation of tumor cells into miniature tumors, and (*ii*) OS cell culture in biomimetic scaffolds [181]. Liquid overlay involves the use of either specially coated (agar-agarose, Poly(2-hydroxyethyl methacrylate) (pHEMA)) or ultra low binding plates that promote the cancer cells sticking to each other as opposed to the substrate [182–190]. The hanging drop technique is widely used [191–194] and involves generating OS cell suspension droplets on the lids of tissue culture vessels, having them sediment, then flipping these lids upside down above media and allowing gravity to instigate aggregate formation and fall into the media [181]. Co-cultures with endothelial cells have also been successfully performed [194]. As with the ESFT, cell adhesion mediated drug resistance associated with the tumor microenvironment was found in OS tumor spheroids [192]. Additionally, tumors generated with this method had fairly uniform and regular edges resulting in a compact configuration associated with native human OS solid tumors, and loss of morphology could easily be observed with exposure to chemotherapeutics making it valuable for drug screening [193].

Biocompatible scaffolding approaches enabled OS cells to interact with biomechanical and biochemical cues that impact tumor formation and migration [181,193,195] and even enable culture of cancer stem cells (Fig. 5D) [196]. For example, nuclear factor-kappa B and the pericellular ECM were found to play critical roles in affecting pulmonary metastatic potential [197,198]; downregulation of the microRNA miR-29b-1 was identified as a novel therapeutic agent [199]; secreted frizzled related protein 2 was found irrelevant to proliferation but important to migratory and invasive potential [200]; the role of vascular endothelial cadherin in transdifferentiation of OS cells to endothelial-like cells, and the ability of the bisphosphonate zoledronic acid to mitigate this process [201, 202].

Quite surprisingly given the native tumor biology, very few OS models are heterotypic [181]. Unlike MM, most of the OS models have used cell lines, mostly metastatic lines of murine origin. One recent effort involving an OS-lung cell co-culture demonstrated that lung endothelial HULEC-5a cells act as attractants for OS cell migration, proliferation, and survival, in an investigation of a distal OS metastatic site [203]. A co-culture of OS cells with MSC-differentiated osteoblasts showed cabozantinib, a tyrosine-protein kinase Met inhibitor, as a new potential treatment for OS [204]. Compared to the other BM tissue engineering fields, there is a number of sophisticated engineered *in vitro* models of OS. Fortunately, advancements made in tissue engineering of the BM microenvironments could be applied towards studying the impact of the BM niche on OS.

Future Directions

Our goal was to discuss advanced strategies to bioengineering of the BM niche in both the healthy and malignant context, and to show how those strategies could be implemented. A necessary step forward would be to develop heterotypic models that more precisely mimic the complexities of the BM niche. An important element missing in many of these models is the *functional vasculature* [59, 94]. The vascularized bone microenvironment plays critical role in BM homeostasis as well as in tumor growth and metastasis. We discussed here the currently available methods for vascularizing bone that have been used for studying MM and breast cancer metastasis [106–109, 146]. However, the fully functional vascular networks with controllable endothelial permeability needed to recapitulate tumor extravasation and intravasation are yet to be developed. In addition, vascularization of primary tumors would allow new biological insights, and the identification of new therapeutic targets [206, 207].

Another critical component missing from all BM malignancy models, which would give them a clear advantage over xenografts, *is the presence of human hematopoietic and immune compartments*. By introducing immune cells, and tracking and quantifying their activity, the tissue-engineered tumor models could become powerful tools for designing and testing strategies for immunotherapy in a physiologically relevant context. Animal models are difficult to work with when trying to investigate the interplay between the cancer cells, hematopoietic, and immune cells [25]. Moreover, the lack of a human immune system makes it very difficult to evaluate checkpoint inhibitors that are becoming increasingly clinically relevant. The potential of 3D models of BM for the development of cellular immunotherapies has been shown in initial studies of MM targeting by αβ-T cells [151].

Osteoclasts that are known to be involved in the maintenance, growth, and drug resistance in various BM malignancies as well as in other bone diseases [207], are another critical HSC-derived component missing from most *in vitro* models. For example, MM cells have been found to release macrophage inflammatory protein (MIP)-1alpha and (MIP)-1beta that enhanced osteoclastic bone resorption [208]. ESFT cells secrete the receptor activator of nuclear factor κB ligand and macrophage-colony stimulating factor that promote osteoclastogenesis [209]. Osteoclasts are also involved in the maintenance of quiescent, drug resistant leukemic cells [210,211]. Our group has shown how osteoclasts can be co-cultured in a BM biomimetic niche [165]. Incorporation of HSPCs and other immune cells could inform novel approaches towards the inclusion of tumor associated macrophages within BM

malignancy models, which would be of clinical value given the implication of these cells in drug resistance and metastasis across BM cancers [212–215].

Not to be overlooked are the improvements that could be made across BM tissue engineering models by better recapitulation of biochemical and biophysical microenvironmental cues. It has been observed that increases in the engineered biomimicry of the native mineralized and ECM BM architecture have led to increased accuracy of *in vitro* models [50,80,111,126,146,181]. Decellularized Wharton's jelly could serve as an excellent mimic of BM ECM in further studies [111–113]. In particular, the decellularized Wharton's jelly allowed for the culture and maintenance ex vivo of chemoresistant leukemic stem cell populations, serving as an effective tool for modeling the BM hematological malignancies like MM.

Another approach pioneered by our group [103–105] and others such as Bourgine et al [80] shows that simply seeding MSCs onto bone-like scaffolds and culturing them in osteogenic media leads to differentiation into functional osteoblasts that subsequently secrete and deposit a BM-like ECM in the surrounding scaffold space (Fig. 2C) [80]. Perhaps unsurprisingly this method also allowed for the most accurate engineered model of HSPC functional compartmentalization to date that could also be applied to malignant BM fields. Alternatively, there are now commercially available decellularized porcine-derived bone ECM gels and scaffolds that could act as more complete analogs of the BM ECM. Additionally, future studies could employ some of the advanced biochemical scaffolding methods capable of fine tuning the physical stiffness and ligand binding densities used, for example, in leukemia modeling towards other BM-related biological questions (Fig. 4A) [139].

Engineering of biophysical stimuli experienced by cells of the BM niches such as fluid flow derived shear stresses and nutrient distributions as well as biomechanical loading that occurs on native bone has already yielded several promising results, suggesting these methods should be integrated into all future BM work. For example, it has already been described how critical perfusion is towards maintaining the dual nature of HSPCs in the BM niche as well as in aiding in ex vivo culture and modeling of chemoresistance in several models of BM maliginancies [80]. Our group additionally showed that the incorporation of mechanical loading can re-activate a pathway for resistance in the ESFT to a class of chemotherapeutics commonly used in treatment (Fig. 5C) [180]. Largely missing from these various models across fields is any sort of standardization towards native-like benchmarks. For example, the perfusion model used successfully by Bourgine et al [80] has a nearly 1,000 fold higher fluid velocity than that used by our group in our perivascular niche on a chip, which was modeled to precisely replicate native human BM interstitial fluid flows (Fig. 3A–C) [80, 109].

In general, with continued improvements in bioengineering of the BM niches, it is becoming increasingly important to shift focus away from ad hoc goals such as maintaining a certain cell population ex vivo, towards a more global perspective properly benchmarked to native human BM. Such a change would require, among other things, an increase in transcriptomic and proteomic sampling of engineered samples, coupled with comparative analysis with native tissues.

In addition to building more heterotypic models with higher biological fidelity to the native BM, another avenue lies in making these models more *patient specific*, to allow improved preclinical drug screening for malignant BM models. Already we have seen techniques employed in patient specific modeling within MM, a disease like osteosarcoma plagued by extreme inter-patient genetic heterogeneity. This was particularly evident with the 3DTEBM tissue-engineered model of MM, where primary BM aspirates were used to derive patient specific BM cells and the matching ECM [146]. Remarkably, this model was able to serve as a highly efficient predictor of clinically relevant drug concentrations [147], showing promise for such an approach to modeling other BM malignancies. The significant limitation of such techniques is their reliance on primary patient materials. In this respect, induced pluripotent stem cells (iPSCs) could be used to overcome these limitations, as well as expand the translational potential of tissue-engineered models. Techniques already exist for the differentiation of iPSCs into a variety of the constituent cell types of the native BM niche: MSCs [216], osteoblasts [217], osteoclasts [218], endothelial cells [219], and HSCs [220]. iPS-derived models have already proven useful in modeling congenital bone disorders [221], and the potential as a renewable, patient specific source of cells for patient-specific studies [222].

Conclusion

In this review we highlight the tissue engineering approaches used to 3 dimensionally model healthy bone marrow and its malignancies. Our goal was to show these methods for the first time in parallel, so that achievements made in one specific BM field like multiple myeloma could shed new insights for the de novo design of the BM microenvironment for another field like osteosarcoma, enabling an interdisciplinary approach towards improved modeling of a niche common to normal and diseased BM.

Abbreviations

3D	three-dimensional
BM	bone marrow
HSPC	hematopoietic stem and progenitor cells
HSC	hematopoietic stem cell
ECM	extracellular matrix
MSC	mesenchymal stem cells
SCF	stem cell factor
PCL	poly(ε-caprolactone)
AML	acute myeloid leukemia
ALL	acute lymphocytic leukemia
LSC	leukemic stem cell

MM	multiple myeloma
ESFT	Ewing's sarcoma family of tumors
ES	Ewing's sarcoma
OS	osteosarcoma
iPSC	induced pluripotent stem cell

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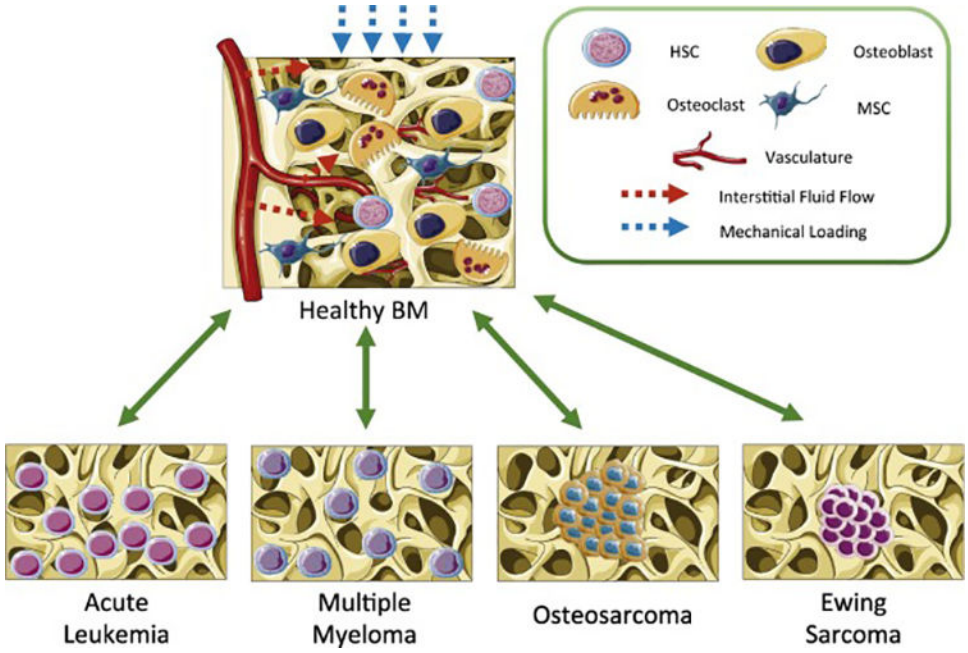

Fig. 1. The bone marrow (BM) as a healthy and malignant tissue.

Normal, functional BM is a niche with complex cell-cell and cell-matrix interactions. Additionally, cells are exposed to a combination of biophysical stimuli such as fluid flow-associated shear stresses and mechanical loading. There are also nutrient and oxygen gradients that develop in an inverse relationship with distance to vasculature. Cancer can develop from either the hematopoietic (leukemia, myeloma) or bone tissue cells (osteosarcoma, Ewing sarcoma) of the BM. Three dimensional tissue engineering approaches have had various success in modeling the various components of healthy and malignant BM. In this review, we will highlight the numerous seemingly disparate models created and offer insights into how discoveries of de novo BM modeling in one field could inform the other fields where the BM is also the host microenvironment.

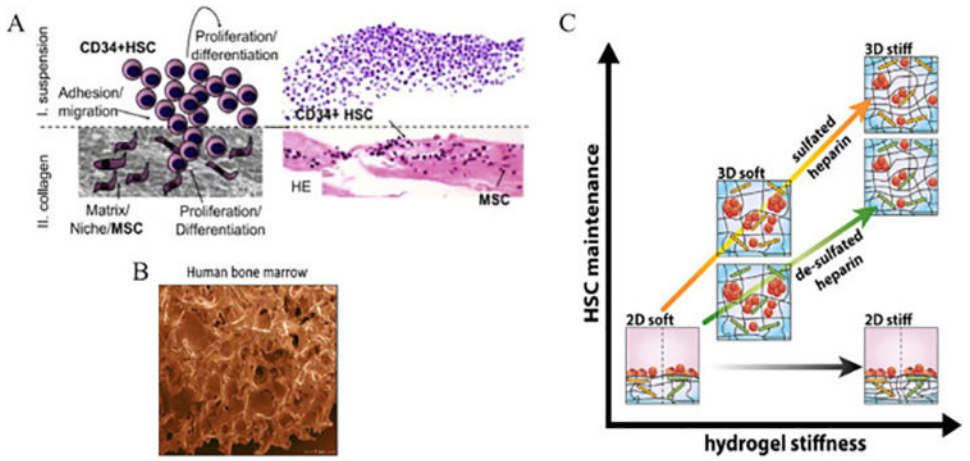

Fig. 2. Tissue engineered modeling of healthy BM.

(A) Co-culture of human hematopoietic stem and progenitor cells (HSPCs) with mesenchymal stem cells (MSCs) in a 3D collagen scaffold demonstrated the formation of 2 specific hematopoietic niches as seen in the schematic and H&E stains. Within the endosteal-like collagen, HSPCs retain their primitive CD34+/CD38- phenotype and clonal growth dynamics, while in suspension above the collagen the HSPCs are much more proliferative and show differentiation. (Adapted from [66]). (B) A hydroxyapatite coated zirconium oxide scaffold mimicking the stiff architecture of trabecular bone was used to co-culture cord blood derived HSPCs and MSCs in the first human “bone-marrow-on-a-chip”. When cultured in a microfluidic device, HSPCs could be maintained in their primitive CD34+/CD38- phenotype for at least 28 days and retained their ability to differentiate into granulocytes, erythrocytes, macrophages, and megakaryocytes. (scale bar = 1 mm, Adapted from [59]). (C) Co-culture of osteogenic cells and HSPCs in a 3D hydroxyapatite-coated ceramic scaffold within a perfused bioreactor system led to compartmentalized phenotypes of HSPCs reflective of those observed *in vivo*: dormant CD34+/CD38- HSPCs within the bone stroma and proliferative differentiating HSPCs being secreted from the bone-mimic scaffold. Importantly, the scaffold featured stromal osteoblastic cells capable of generating a native-like BM ECM consisting of collagen types 1 and 4, fibronectin, and osteocalcin. (scale bar = 100 μm, Adapted from [80]). (D) A novel, modular, glycosaminoglycan (GAG)-based biohybrid hydrogel system capable of simultaneous manipulation of the exogenous biochemical and biophysical signals was used to mimic the dynamic BM niche where a balanced population of proliferative and quiescent HSPCs is maintained. It was found that increased GAG concentration and reduced spatial confinement as a result of increased hydrogel stiffness caused a reduction of HSPC proliferation and cell cycling, yielding larger quiescent cell populations. (Adapted from [91]).

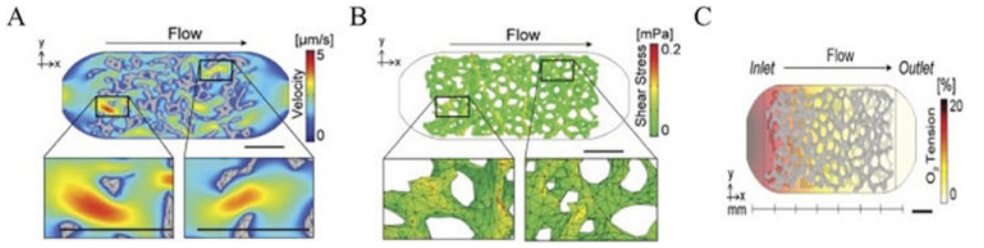

Fig. 3. Modeling of the vascularized BM niche and incorporation of *in vivo*-like fluid flow.

A human bone perivascular "niche-on-a-chip" was developed by seeding BM-derived MSCs and human umbilical vein-derived endothelial cells in a de-cellularized trabecular bone scaffold and culturing in a microfluidic chip where the tissue chip is exposed to perfusion mimicking native interstitial fluid flow. The microfluidic chip enables tight control over flow velocities (A), shear stresses (B), and oxygen gradients (C). As a result, a long-lasting, self-assembled vascular network with surrounding perivascular-like cells could form within the bone scaffold without the addition of angiogenic factors typically needed in such co-cultures. (scale bar = 8 mm, (Adapted from [109]).

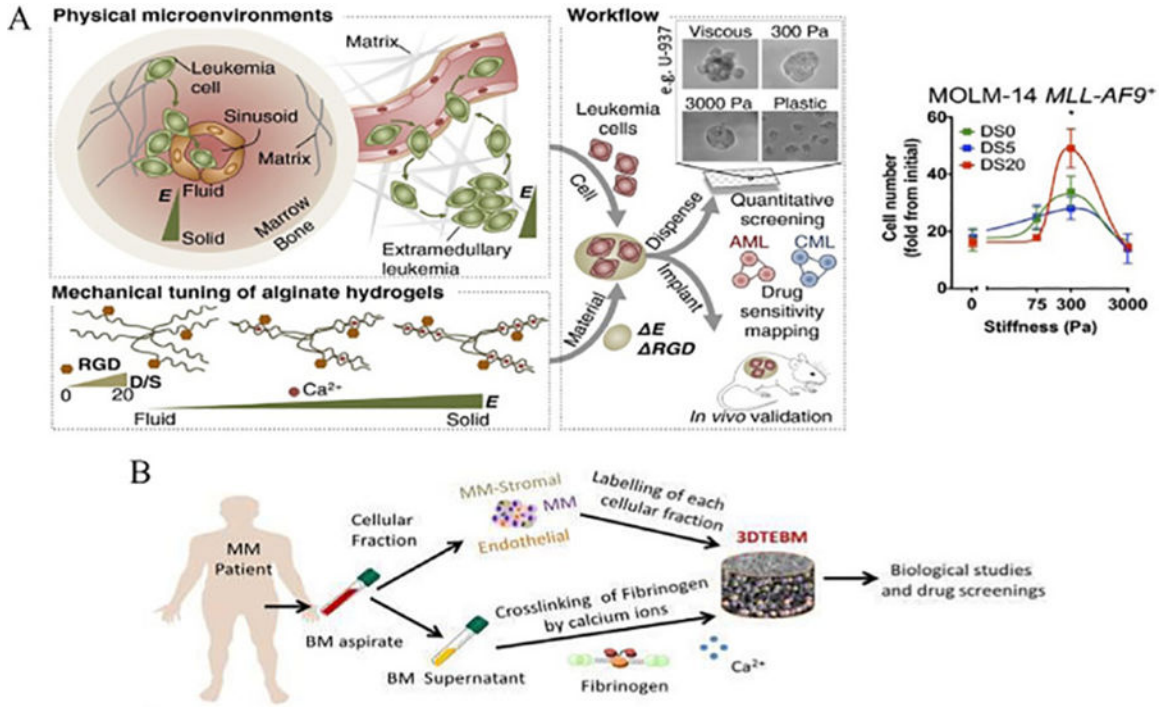

Fig. 4. 3D modeling of hematologic cancers within their BM niche.

(A) Tissue engineering of the leukemic BM niche and modeling the 3D microenvironment demonstrates effects on cancer cell growth and chemoresistance. A 3D alginate hydrogel with easily manipulated matrix stiffness and Arg-Gly-Asp (RGD) integrin ligand density was used to mimic the varied nature of the BM niche (left). A variety of leukemias and 3D matrices were screened *in vitro* against clinically relevant chemotherapeutics followed by *in vivo* valdiation (center). Cell proliferation was variably regulated by both stiffness and ligand density in a cell-line dependent manner (right). Adjustment of these mechanical properties, and specifically matrix softening, resulted in increases in drug resistance (Adopted from [139]). (B) Development of a patient specific 3D-tissue engineered model of multiple myeloma (MM) to study pathogenic biology and identify sources of drug resistance. BM aspirates were obtained from MM and used to isolate both the cellular fraction of MM (stromal, endothelial, and MM cells) and matrix fraction (BM-derived fibrinogen) which were recombined within an ionically cross-linked native matrix hydrogel (3DTEBM). This model promotes MM proliferation ex vivo, allows for cell-cell interaction with surrounding BM niche cells, recreates oxygen and drug concentration gradients observed *in vivo*, and confers increased drug resistance as compared to traditional 2D *in vitro* cell cultures of MM (Adopted from [146]).

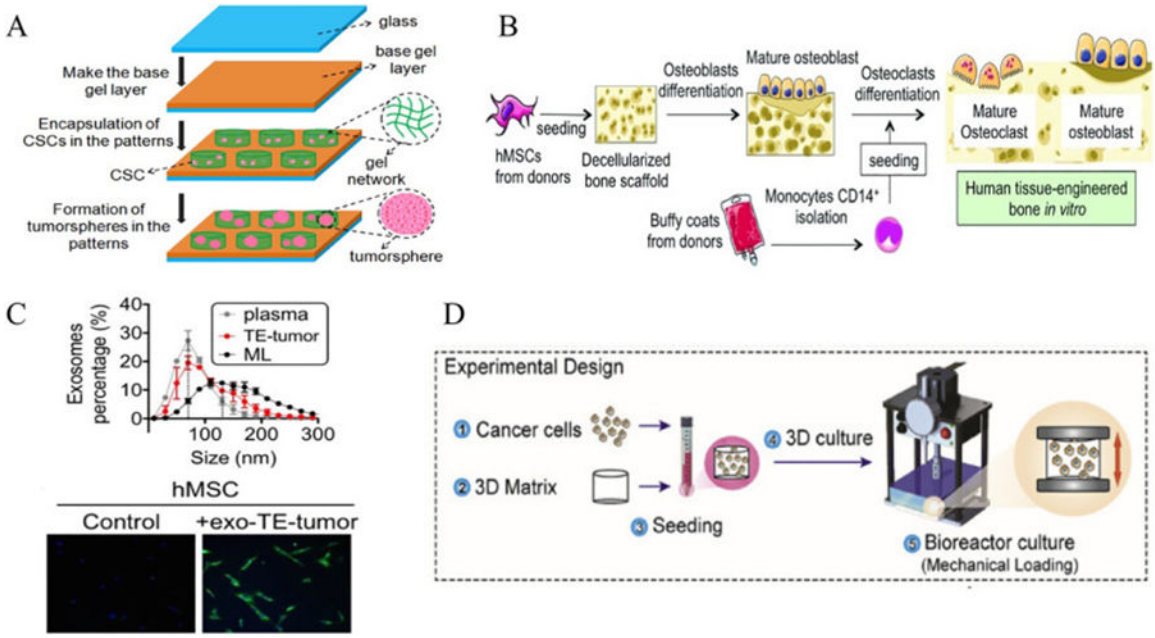

Fig. 5. Tissue engineering approaches to mimic primary bone tumors within their native BM niche.

(A) In order to better recreate the BM niche of primary bone tumors, our group developed a protocol for the co-culture of functional bone-depositing osteoblasts and bone resorbing osteoclasts within a trabecullar bone scaffold. Ewing sarcoma tumor aggregate introduction results in decreases to bone density, connectivity, and matrix deposition, as observed clinically (Adopted from [165]). (B) The importance of 3D culture of primary bone tumors within tissue engineered BM niche models is evident from exosome analysis: only cancers grown in this context can recapitulate the exosome size (top) and mRNA cargo observed in patients. Furthermore, exosomes can be labeled fluorescently, and their interactions with cells of the surrounding niche observed microscopically (bottom) (Adopted from [171]). (C) A bioreactor was designed to mimic mechanical loading on the BM niche like that observed *in vivo* and was used to study mechanical signaling on Ewing sarcoma cells within a collagen 3D matrix. Compressive forces caused a restoration of the ERK1/2-RUNX2 pathway and resulted in increased drug resistance (Adopted from [180]). (D) Osteosarcoma cell lines, along with others, were grown into tumorspheres within polyethylene glycol diacrylate (PEGDA) hydrogels featuring a compressive moduli range of 2–70 kPa. Generally speaking, two main approaches are used in sarcoma studies: generation of tumor aggregates or growth of cells with hydrogels, This system allows for both, and was used to show a connection between the YAP/TAZ transcription factors, matrix stiffness, and sarcoma cell growth. (Adopted from [198]).