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3D models of the bone marrow in health and disease: yesterday, today and tomorrow

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Abstract

The complex interaction between hematopoietic stem cells (HSCs) and their microenvironment in the human bone marrow ensures a life-long blood production by balancing stem cell maintenance and differentiation. This so-called HSC niche can be disturbed by malignant diseases.

Investigating their consequences on hematopoiesis requires deep understanding of how the niches function in health and disease. To facilitate this, biomimetic models of the bone marrow are needed to analyse HSC maintenance and hematopoiesis under steady-state and diseased conditions. Here, 3D bone marrow models, their fabrication methods (including 3D bioprinting) and implementations recapturing bone marrow functions in health and diseases, are presented.

Keywords

Biological; bone; biomedical

Introduction

The red bone marrow, located in the cavities of the trabecular regions in long bones, represents a complex blood and bone cell-producing organ. Blood cells develop from immature hematopoietic stem and progenitor cells (HSPCs) and ensure the completion of several essential tasks like immune function, blood clotting or oxygen transport. Whereas most mature blood cells undertake their tasks mainly outside of the bone marrow, the only place where HSCs execute their functions (stem cell maintenance and blood cell production by differentiation) to guarantee lifelong blood production, is in their stem cell niche in the red part of the bone marrow.[] Current studies revealed the existence of such niches at certain sites of the bone marrow. These niches are illustrated in a simplified form in Fig. 1. One of those is described as the endosteal niche, characterized by bone regenerating osteoblasts (OBs) located at the endosteal surface of bone.[] OBs are able to regulate HSC function e.g. by activation of the Notch pathway and production of thrombopoietin (TPO), they are involved in controlling HSCs quiescence.[,] Another site for HSC niches was

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described close to the vascular structure near the endosteum, specified as arterioles, which have also an impact on HSC quiescence and thus form a unique arteriolar niche.[] Nevertheless, dividing and non-dividing HSCs are mainly found in the perisinusoidal regions of the bone marrow.[] Here, HSCs are in close proximity to blood vessel covering endothelial cells, which affect HSC dormancy and self-renewal via cell-cell contacts (e.g. via E-selectin). This region is generally referred to as the endothelial/perisinusoidal niche.[,] At this perivascular site, HSCs are in close contact with enriched mesenchymal stromal cells (MSCs) which are subdivided into Leptin⁺, C-X-C motif chemokine 12 (CXCL12) abundant reticular (CAR) or Nestin⁺ cells. These cell populations are an important source of factors required for HSC maintenance.[,] Apart from these, hematopoietic cells which are found throughout the bone marrow niches, are also able to influence HSC fate.[] Among others, T-cells in the endosteal region regulate stem cell maintenance, whereas an ablation of megakaryocytes, the early precursor of blood clotting thrombocytes, from the bone marrow results in mobilization of HSCs into the peripheral blood system.[,] The named endothelial, perivascular or hematopoietic cells are just a selection out of many niche cells, which are all known to regulate HSC function and are important regulators in the environment of HSCs. The niche cells govern HSCs by providing cell-cell contacts or by production of soluble factors. The most popular cell-cell contacts in the context of the HSC niche are cell-cell adhesions via N-cadherin, interactions via vascular cell adhesion molecule 1 (VCAM1) and very late antigen-4 (VLA-4; integrin $\alpha 4\beta 1$) or the notch ligand-receptor interplay.[,] In addition to these, soluble factors such as fibroblast growth factor 2, WNT ligands, stem cell factor (SCF) or CXCL12 expressed by MSCs as well as bone morphogenic proteins (BMPs) or angiopoietin expressed by other niche cells have an impact on cell cycle, maintenance of HSCs and normal niche function.[] Furthermore, niche cells produce extracellular matrix (ECM) components. The ECM, embedding the cells in their niche, consists of proteins such as collagen IV, glycoproteins such as fibronectin or glycosaminoglycans (GAGs, e.g. hyaluronic acid (HA)) and matrix remodeling enzymes. This protective shelter functions as a growth factor reservoir and determines the balance between stemness and differentiation with the help of cell-matrix interactions.[-] HSCs can bind to ECM proteins mainly via membrane-bound integrins.[] On one hand, this interaction can result in a protein sequence-specific cell response; on the other hand the cells can sense biophysical properties of the substrate leading to a substrate stiffness-dependent cell response. In the latter case, HSCs rearrange their cytoskeleton after substrate binding and exert traction to the substrate. In a process called mechanotransduction, this mechanical stimulus can be converted into a biochemical signal in the cell, which can influence HSC behavior.[] The stiffness in the bone marrow affecting HSC behavior ranges from 0.1-1 kPa in the central marrow and 5-20 kPa in the perivascular region up to >35 kPa in the endosteal part.[]

This summarized overview of bone marrow components exhibits that HSCs are embedded in an intricate environment in which they receive cell-specific, biochemical and biophysical signals from all three dimensions. This unique environment of the bone marrow represents the only place where HSCs are able to maintain their stem cell properties life-long. So far, *in vitro* culturing of HSCs is not sufficient to maintain stem cell properties and ends up with differentiation of the stem cells into committed progenitors or mature blood cells. Sufficiently *in vitro* multiplied HSCs from one donor could be used for cell transplantations

into several patients with leukemia and thereby overcome the problem of donor limitations.[] Furthermore, malignant diseases concerning the bone marrow can severely alter or even destruct the complex interaction in the HSC niche.[,] The treatment of such disorders comprise the medication with disease-specialized drugs.[] For effective targeting of the disease and to improve the therapeutic outcome new drugs have to be developed. To achieve these goals, more information on how biochemical and biophysical cues regulate HSC fate and how these signal transductions are changed in a diseased bone marrow, are required.

In order to cope with these scientific tasks, bone marrow model systems are needed which i) allow analysis of human hematopoiesis in steady-state and disease, ii) can be used as drug-testing systems and iii) facilitate the *in vitro* expansion of HSCs. For this purpose, animal models are often used, accepting the major drawback of insufficient transferability to human beings.[] Furthermore, the ethical aspects and in this context the implementation of the 3Rs principle to replace, reduce and refine animal experiments urges alternatives.[] Therefore, *in vitro* models have steadily been developed during the last years. The usage of conventional 2D cell culture devices is becoming less common due to the increasing knowledge of the *in vivo* conditions revealing several drawbacks of the highly artificial 2D environment. The complex interplay of cells with their surrounding in a 3D architecture affects the morphology of stem cells as well as their differentiation into mature cells disclosing the importance of a 3D environment for HSCs.[,] In the following chapters, we review state-of-the-art 3D cell culture models mimicking the bone marrow *in vivo* in health and disease as well as methods for their construction with special emphasis on 3D bioprinting. Furthermore, their implementation for *in vitro* cell analysis and future aspects in context to refine bone marrow models are discussed.

Evolution of bone marrow models

As already mentioned, improvements of the HSC expansion for clinical needs and of bone marrow models for fundamental studies and drug-testing are indispensable in future research. To reach this goal, HSC cultures which mimic relevant characteristics of their natural healthy niche in the bone marrow, need to be developed. The evolution of such bone marrow models, which comprised more and more aspects of a natural niche and increased in complexity in the last decades, is described in the following and summarized in Fig. 2. Conventionally, suspension culture systems are used to expand umbilical cord blood HSPCs in research or in clinical studies before transplantations. By adding cytokines to the culture medium, which is drafted in Fig. 2(I), HSC expansion could be improved significantly. Commonly used cytokines for HSC expansion are for example TPO, FMS-like tyrosine kinase 3 ligand (Flt3L), SCF, granulocyte colony-stimulating factor (G-CSF), interleukin-6 (IL-6) and interleukin-3 (IL-3). Furthermore, several developmental regulators such as the notch ligand Delta-1 or small molecules such as the copper chelator tetraethylenepentamine (TEPA), nicotinamide and StemRegenin-1 were used in addition to the cytokine cocktails, which further improved the total cell and CD34⁺ HSPC expansion.[] Likewise, several cell types that occur naturally in the bone marrow were described to assist HSC proliferation and maintenance by secreting HSC-supporting factors in cell cultures, which is delineated in Fig. 2(II). This includes for example OBs, naturally present in the endosteal niche, endothelial cells, *in vivo* an important cell type of the vascular niche, and others that were mentioned

before in the introduction. The most commonly used supporting cells in HSC cultures are MSCs because they express higher levels of HSC-supporting factors than other stromal cells, [] and they were already used to expand HSCs for transplantations in clinical studies. In clinical studies, neutrophil and platelet recovery were slightly improved in patients with previous *in vitro* HSPC expansion before transplantations compared to direct transplantations without HSPC expansion. However, no significant improvement in survival of the patients was reported, as recently reviewed.[] Conventionally used protocols supported the proliferation of HSCs but they did not foster the self-renewal of HSCs to a large extent, which led to a rapid decline of stemness in such *in vitro* cultures.[] With the purpose to improve the expansion of HSCs while maintaining stemness, researchers looked more closely at the natural stem cell niche and started trials to mimic it in its entirety, including chemical conditions, cell and ECM compositions as well as biophysical properties.

In this attempt, researchers tried to imitate the ECM of the bone marrow in many different ways. In standard cell culture, conventional tissue culture plates, which are made from polystyrene and are thus stiff ($E=3,000$ MPa []), were used to culture HSCs in suspension. By coating the culture devices with natural components of the bone marrow ECM, such as fibronectin or collagen, as shown in green in Fig 2(III), HSCs were enabled to attach to this matrix. The attachment of HSCs does not only affect the binding itself but also influences proliferation and stemness of the cell e.g. by mechanotransduction, which is why it is important to allow attachment of the HSCs also in *in vitro* cultures. Instead of proteins, also smaller bioactive sequences, such as the minimal integrin recognition motif RGD or the fibronectin fragment CS-1, were applied to allow the attachment of the cell to synthetic matrices. Placing these proteins and peptides in a controlled manner by nanopatterning added another biophysical parameter to culture systems, since nanoscale topography and nanopatterning also have effects on cell behavior. For example, scientists found out that a distance less than 45 nm between cyclic RGD peptides is required for proper HSC attachment and that efficient cell-peptide adhesion may foster proliferation and differentiation of HSCs.[] Another physical parameter, which influences stem cell behavior, is the stiffness of the substrate. Cells can track the stiffness of a substrate by mechanosensing and thereupon change their behavior. For example, when MSCs were grown on a stiff substrate, they differentiated and expressed markers of osteogenic lineages whereas on soft surfaces they expressed neurogenic lineage markers.[] For HSPCs it was found that they adhered better, were more motile and kept a higher level of stemness when they were cultured on stiffer hydrogels.[] They proliferated the most if they were cultured on surfaces with intermediate stiffness (approximately 12.2 kPa).[]

In an *in vitro* 2D culture it is almost impossible to reciprocate complex biochemical and biophysical aspects as they can be generated in 3D *in vitro* cultures. For instance, the 2D environment does not mimic the 3D bone marrow *in vivo* in architecture, leading to an irrelevant phenotype of cells.[] In contrast to a 3D culture, cells in 2D grow in an unnatural dorsal-ventral polarization affecting their contact surface for cell-cell or cell-matrix interactions, which in turn influences cell morphology. For example, MSCs flattened when cultured on 2D surfaces, which further affected the tension in the MSC cytoskeleton and the expression of pluripotency genes.[] On top of that, cells in 2D culture are covered by large volumes of medium. Secreted molecules are diluted immediately in the medium and no

signal gradients can be created which impairs autocrine and paracrine signaling.[1] Due to these drawbacks, the idea came up to also consider dimensionality in cell culturing.[1] Many different 3D cell culture systems with different complexity were developed and found to support HSC expansion and maintenance to a higher level as 2D systems. In the following part, some of them will be presented.

The simplest 3D *in vitro* cultures are spheroids. To generate spheroids, cells can be cultured in hanging drops or on non-adhesive surfaces to force them to form aggregates. In Fig. 2 (IV) a hanging drop culture is sketched, comprising an aggregate of different cell types within a drop of medium. An example is the high-throughput coculture system with MSCs and HSCs presented by Cook and colleagues.[1] They used surface modified microwells to support the formation of microaggregates, which then developed spatial structures. It could be observed that, in comparison to 2D cultures, MSCs in the aggregates expressed higher levels of hematopoietic niche factors and the HSC expansion increased. Such spheroids have the advantages that the cells can form proper cell-cell contacts and tissue-like patterns, they keep their natural shape, signaling molecules are not diluted into the medium (except at the spheroid-medium interface) and thus cells can communicate properly.[1] Currently, a widespread method to culture HSCs in 3D is the incorporation of cells into a polymer solution that is cross-linked to form a gel (depicted in green in the second part of Fig. 2(IV)) in the following. The cells are encapsulated in the forming hydrogel, which resembles the ECM *in vivo*. Natural or synthetic polymers, which are able to swell in water, were used for this purpose. Materials such as fibronectin, collagen, laminin and GAGs occur in the bone marrow *in vivo* and HSCs attach easily to them, which is why they were often used in 3D cell cultures.[1] Other natural materials used for scaffolds were chitosan, alginate, Matrigel™ (gelatinous protein mixture), agarose and bacterial nanocellulose.[1, 2] By functionalizing synthetic matrices with small peptides, such as RGD, the culture conditions can be controlled precisely, while native ECM molecules, such as HA, not only affect adhesion, but also influence a lot of other signaling pathways.[1] Conventional synthetic polymers used for hydrogels are, for example poly (lactic acid) (PLA), poly (ε-caprolactone) (PCL), poly (lactic-co-glycolic acid) (PLGA) and poly (ethylene glycol) (PEG). These materials were used alone or in conjunction with each other. In some models, inorganic components of the bone such as hydroxyapatite (HAP) and tricalcium phosphate (TCP) were also incorporated. [1, 2] Proper attachment of cells and cell compatibility are major requirements of these gels and the possibility to modulate the gel properties, such as stiffness, is a further advantage to improve them as culture systems. For the further usage of HSCs, e.g. in transplantations, it is important that the cells can be efficiently recovered from the gels without harming them. This requirement could be met by degradable gels. Gels with natural polymers can, for example, be degraded by enzymes whereas in synthetic materials special cross-linkers (e.g. polymers with disulfide bonds), which are cleaved by special stimuli such as light (photocleavable linkers, e.g. Fairbanks et al.[1]) or reducing agents (e.g. Zhang et al.[1]) can be introduced.[1] Such stimuli-responsive gels gain more and more importance nowadays. An interesting application is the usage of biodegradable hydrogels. Cytokines, small molecules or other factors that influence cells are incorporated into the gel and undergo a sustained release during the process of degradation induced by the cells themselves (e.g. Cheng et al. [1]). Also cell migration, cell-cell contacts and stemness of the cells was improved, if cells

were able to remodel their matrix.[] The advantages of encapsulation are that cells have full contact to the matrix and that signaling molecules are not diluted quickly. However, the cells are often not in direct contact. Since the natural bone marrow does not consist of a homogeneous matrix but is perforated by pores, meshworks made of fibers and porous sponge-like scaffolds, such as the sketch in the third part of Fig. 2(IV), were developed to mimic this architecture. The cells were added after scaffold preparation. Thus, in these approaches the cells were not directly surrounded by the gel, but they were gathered in the pores of the gel. This permitted the formation of close cell-cell contacts and a 3D architecture for cell-matrix contacts.[] The pores could be generated by different methods, such as salt leaching, freeze-drying or 3D printing and by changing parameters of the manufacturing protocols, pore sizes and shapes could be varied. To mimic the *in vivo* conditions and to provide an adequate architecture for 3D organization of the cells, while allowing for enough place for nutrient exchange, pore sizes between 70 and 300 μm were chosen.[] A full interconnectivity of the pores was achieved, so that the medium and the cells could completely penetrate the scaffold. Examples for successful applications of porous 3D hydrogels for the expansion of HSPCs *in vitro* are the scaffolds of Ferreira et al. [] and Raic et al. []. Ferreira et al. tested several natural polymers as scaffolds in combination with either cytokines or MSCs as support and found fibrin in combination with MSCs to be the most promising system with a 3×10^7 -fold expansion of CD34⁺ cells compared to conventional 2D systems.[] Fig. 3(a) shows a scanning electron micrograph of HSCs and MSCs attaching to a synthetic poly (ethylene glycol) diacrylate (PEGDA)-RGD scaffold. Raic et al. showed that HSPC expansion was upregulated in this scaffold in comparison to the expansion in 2D tissue culture plates if HSPCs were cocultured with MSCs.[]

To culture large amounts of cells in an active state over a long period of time, bioreactors were used. Here, we use the term bioreactor to describe an engineered system that allows automated control over medium supply, waste removal and agitation. Bioreactors were combined with 2D or 3D applications. Rödling et al. developed a bioreactor containing a PEGDA-RGD scaffold, which resembles the drawing in Fig. 2(V). It could mimic the niche in an active or in a steady-state condition that either supported HSPC differentiation or HSPC maintenance.[] Microfluidic devices can also accomplish a continuous nutrient and oxygen supply and, furthermore, gradients (e.g. oxygen, calcium ions, cytokines and small molecules) can be generated.[] Such systems operate with extremely small amounts of fluids (10^{-6} to 10^{-15} mL) using laminar flow microchannels (1-1000 μm). To mimic blood vessels that are pervading the tissue *in vivo*, microfluidic devices were used and in this way another important parameter of the bone marrow – the mimicry of vasculature and perfusion – could be included into models. Kotha et al. developed a sophisticated model that mimics very well the vascular niche and the process of HSC homing in a microfluidic device, which comprises various cell types, a flow system and a soft matrix. [] They constructed an endothelialized vascular network in a collagen matrix with MSCs and other bone marrow stromal cells. After perfusing the network with medium containing monocytes (CD14⁺/CD45⁺) or HSPCs (CD34⁺/CD45⁺), they could observe these cells adhering to or migrating into the soft matrix. Very complex microfluidic systems are organs-on-a-chip. An interesting example is the bone marrow-on-a-chip from Torisawa and colleagues.[] They implanted a collagen gel with demineralized bone powder and BMP subcutaneously into a mouse where it developed into

a bone with an intact bone marrow. Afterwards, the bone was taken out and put into a microfluidic device where it could be cultured for at least one week. This system proved to be a proper *in vitro* model for irradiation toxicity tests. However, it was of murine origin and did thus not represent the human bone marrow. Reinisch et al. developed humanized bone marrow-ossicles by implanting human MSCs into mice and let them form bones, which were then chimeric, of murine and human origin.[] Such microfluidic systems are also one of many possible approaches to mimic the bone marrow in malignant diseases by imitating the invasion of cancer cells from the blood stream into the bone marrow *in vitro*. In the following two chapters, such approaches for models of leukemia and bone metastasis will be presented and discussed.

Leukemic niche models

In hematological disorders such as leukemia the bone marrow is overflowed by malignant hematopoietic cells leading to a disturbed or insufficient hematopoiesis.[] Transformation of hematopoietic cells into malignant cells can occur due to genetic events, hereditary or environmental conditions and affect the myeloid or lymphoid cell lineage.[,] A small proportion of these cells regain stem cell properties, such as an unlimited self-renewal potential and the ability to initiate leukemia as well as a reduced differentiation potential.[,] The malignant leukemic cells (LCs) occupy the bone marrow niche for their own survival and an uncontrolled proliferation contributing to disease progression.[,] The highly organized HSC niche is rearranged by LCs and a leukemic niche is formed, accompanied by a loss of important signals and niche cells, impairing normal homeostasis.[] In this protective environment LCs are not assailable for some therapeutic procedures aiming at the destruction of malignant cells.[] Due to their acquired stem cell properties and the interception of anti-apoptotic signals, they can survive in the marrow despite harsh conditions during treatment and can lead to a reappearance of the disease.[, ,] Development of new therapeutic strategies to address this problem needs *in vitro* tools overcoming the limitations of animal models, in most cases mouse-based model systems.[] Apart from the obvious discrepancies between human and mice, such as bodyweight, lifespan or fertility, the differences in assembly of the peritumoral milieu limit the often used xenotransplantation models which allow the investigation of human cells in an *in vivo* system.[–] Therefore, *in vitro* models gain more and more importance in investigating the origin and the progression of hematopoietic malignancies and the associated signaling pathways to develop new treatment strategies. For this purpose, current studies are focused on the establishment of *in vitro* models which allow analysis of cell-cell and cell-matrix interactions as well as the interplay between LCs and soluble factors in a 3D environment resembling the *in vivo* conditions. In the following part, *in vitro* models are presented that allow investigations of these regulatory cell interactions and contribute new hints about the function of the human leukemic bone marrow. Similar to HSCs, LCs are also found in the vascular or endosteal part of the bone marrow and can interact with cell-populations of these microenvironments.[,] Bray et al. constructed a 3D PEG-heparin hydrogel with human umbilical vein endothelial cells (HUVECs) amongst others as environmental stimuli to investigate the impact of the vascular niche in acute myeloid leukemia (AML). They found that cells from the vascular network in a 3D scaffold support the resistance of LCs against

chemotherapeutics.[] Furthermore, Kotha and colleagues also developed an artificial vascular microenvironment which allowed studying 3D multicellular interactions and cell trafficking between distinct fibroblasts and LCs. In the *in vitro* vascular marrow, they showed that LCs respond to distinct fibroblasts which were embedded in a collagen type I matrix around the vessels with increased adhesion and migration within the vessel network which is shown in Fig. 3(b).[] In an engineered microfluidic 3D collagen matrix, presenting an endosteal *in vitro* platform, Bruce and colleagues showed direct cell-cell interactions in 3D between MSCs and *in vivo* endosteally located OBs with LCs from acute lymphoblastic leukemia.[] Using *in vitro* transwell filter devices revealed an influence of MSCs, which represent the main population in bone marrow stroma, on survival and viability of LCs isolated from patients with chronic lymphatic leukemia. In addition, they found that a coculture of MSCs with leukemic B cells promotes the cytokine production of MSCs, concluding that LCs are able to rearrange their cytokine milieu.[] These extracellular communication molecules are important for cell homing, migration and growth. Changing their composition by increasing the expression of homing or anti-apoptotic factors in the bone marrow can support LCs survival.[,] Therefore, studying cell behavior in response to soluble molecules is of great importance to find new treatment strategies that are based on abrogating the connection between LCs and their supportive environment. With the objective of finding molecular mechanisms to reach this goal, migration studies in a 3D filter system revealed the involvement of the Raf/MEK/ERK1/2 pathway in the cell response of leukemic T cells after binding of the CXCL12 homing factor. [] CXCL12 is known to mediate chemoresistance after binding to its receptor C-X-C chemokine receptor type 4 (CXCR4) on the cell surface of LCs in different leukemia types.[,] Due to its great importance several 3D cell culture platforms were developed to analyze the impact of the CXCL12/CXCR4 axis on LCs migration. Promising *in vitro* studies concerning disruption of these interactions showed a decreased drug resistance of chronic myeloid leukemia (CML) cells after pretreatment with a CXCR4 inhibitor.[] The combination of a CXCL12 inhibitor and a tyrosine kinase inhibitor reduced cell migration to a greater extent than either drug alone.[] In the endosteal region of the bone marrow, OBs express osteopontin which can bind to its receptor on LCs leading to protection against apoptosis. In a 3D polystyrene scaffold coated with OBs and LCs, the addition of an inhibitor for the osteopontin receptor together with a tyrosine kinase inhibitor disrupted the adhesion between the LCs and the protective microenvironment and increased their sensitivity to drugs. Therefore, blocking the LC-binding to osteopontin could be an efficient therapy for patients with AML.[] Cells encapsulated in a 3D surrounding not only communicate with other niche cells and their expressed cytokines, but also their interactions with the ECM allocate cell behavior. Porous scaffolds fabricated from polymeric materials coated with the ECM protein collagen were proven to be suitable for the culture of leukemic cell lines in contrast to uncoated controls, highlighting the importance of cell-ECM interactions in cell growth.[] Furthermore, migration experiments in a 3D transwell assembly showed an improved migration of a leukemic myeloid cell line during culture with collagen IV.[] Investigations of matrix mechanics and its role on LCs revealed that matrix stiffness of 3D alginate hydrogels regulates proliferation of AML cell lines through autocrine signaling. In addition, matrix stiffness as well as the integrin ligand (RGD) functionalized scaffolds modulated resistance of AML cells against protein kinase inhibiting drugs.[] 3D alginate hydrogels, mimicking the soft marrow, supported cell growth to a

higher extent than the 2D control, led to more diverse myeloid progenitor cells and had an impact on cell cycle.[] The discussed studies indicate that the bone marrow microenvironment including cell-cell, and cell-matrix contacts as well as soluble factors plays a critical role in leukemia progression and survival and protects the malignant cells against chemotherapeutic treatment. Results from a previous study, showing that the drug sensitivity of LCs depended on dimensionality of the environment, further stress the need of new 3D *in vitro* cell culture platforms.[] Rödling et al. developed a reactor system for perfusion of 3D scaffolds mimicking the bone marrow *in vivo*. They could demonstrate the relevance of perfusion during drug treatment as results differed with and without perfusion.[] Therefore, using 3D systems can not only provide new information about the regulation of LCs in a *in vivo* like environment, but is also a promising tool as drug-testing platform to find new therapeutic strategies for patients with leukemia.

Bone metastasis models

Hematological malignancies such as leukemia, lymphoma and multiple myeloma are not the only cancers affecting the bone. Cancer may arise in any organ of the human body. However, it is the metastases, rather than the primary tumor, that result in majority of the cancer-related deaths.[] Metastasis is the multistage progression of cancer cells from the primary tumor to other organs, which, despite extensive research, remains the most enigmatic phenomenon in cancer pathophysiology.[,] In fact, bone occupies the third position among the metastatic sites, briefly trailing behind lungs and liver.[]

Metastasis is a complex series of events beginning with the detachment of cancer cells from the primary tumor and entry into the blood and lymph vessels (intravasation). These circulating tumor cells translocate to secondary organs, fighting immune resistance and tolerating mechanical forces in the process. At this point, they exit from the bloodstream (extravasation), settle in and adapt to the new microenvironment. Now, the disseminated tumor cells may either enter dormancy as single cells or as small clusters (micrometastases), during which they develop therapeutic resistances and long-term survival strategies. The disseminated tumor cells deploy survival signals which give rise to drug resistant clones and finally proceed to form macrometastases.[, ,] Events behind metastasis, such as invasion of circulating tumor cells, growth and survival of disseminated tumor cells, evasion of host-tissue defenses and amplification of survival signals is not only dependent on the innate characteristics of the primary tumor but also on the secondary tissue microenvironment. Different types of cancer show organ-specific metastasis, simultaneously or sequentially. Prostate cancer predominantly spreads to the bone, breast cancer to bone, liver, brain and lungs and colorectal cancer begins with liver metastasis and moves on to lungs and brain.[,]

About 70% of breast and prostate cancers lead to skeletal metastases, followed by thyroid, lungs, kidney and bladder carcinomas.[,] Multiple myeloma can also be considered as a bone metastatic disease as it not only originates in the bone but also spreads throughout the bone marrow.[, ,] Bone metastasis has an extremely poor prognosis and is mainly characterized by severe chronic pain, impaired mobility, hypercalcemia, spinal cord compression, pathological fractures and bone marrow aplasia. Appearance of these

symptoms significantly decreases the quality of life of the patient and, in most cases, results in death.[, ,]

So, what is the reason behind such organ-specific metastasis? In 1889, Dr. Stephan Paget, an English surgeon, put forward his 'seed and soil' hypothesis, shedding some light on this phenomenon. He proposed that specific organs provide a comfortable home or the 'soil' to certain tumor cells, the 'seed'. Progression of metastatic colonization occurs only when 'the seed' has found its hospitable 'soil'.[] The bone marrow is a highly vascularized tissue possessing a rich milieu of cytokines, chemokines and growth factors. It also harbors an arsenal of different cell types such as immune cells, MSCs, HSPCs, bone-forming OBs and bone-resorbing osteoclasts, which express specific molecules that facilitate tumor invasion.[] Kang and colleagues showed that osteolytic factor IL-11, connective tissue growth factor, membrane receptor/adhesive proteins CXCR4 and osteopontin as well as the metalloproteinase1 (MMP1) are important parameters that lead breast cancer cells to metastasize to bone.[]

Stem cell niches present in the bone marrow are of particular interest to the cancer cells. Shiozawa et al. presented that prostate cancer cells and HSCs actively compete with each other for the occupation of endosteal niches. Bone marrow stromal cells and OBs express adhesion molecules annexin A2 (AXA2) and CXCL12 and facilitate the CXCR4 and the AXA2-expressing cancer cells and HSCs to bind and home to the bone marrow. When the cancer cells succeed in hijacking the niche, they force the HSCs to differentiate terminally.[]

Such settings provide the perfect hotbed for the cancer cells to invade, thrive and expand. The bone always undergoes active remodeling which requires a strict balance between OBs and osteoclasts. However, during metastatic colonization this equilibrium is disrupted and leads to abnormal increase in either cell type.[] Osteolytic lesions are usually common in breast cancer and multiple myeloma whereas prostate cancers are more prone to osteoblastic metastasis.[,] This is why, it is important to investigate cancer metastasis in a bone marrow microenvironment.

In general, tumor microenvironment (TME) is the amalgamation of all cellular and extracellular components surrounding cancer cells with which they maintain an effective crosstalk. Cancer-associated fibroblasts, transformed epithelial cells, tumor infiltrating MSCs, immune cells, adipocytes, and endothelial cells of blood and lymphatic systems are some of the cellular constituents of the TME. Cadherins are the most important molecules aiding interactions among the cellular components whereas cell-matrix interactions are usually established by integrins present on the cells. In the TME, various growth factor- and cytokine-mediated signalings take place, in an autocrine or a paracrine manner.[–] Such interactions with the tumor cells influence various biological characteristics such as migration, proliferation, quiescence, immune and therapeutic resistances. The presence of a physicochemical gradient in an avascular tumor mass not only limits the access of oxygen, nutrients and soluble factors but also alters the diffusion profile of drugs into the tumor.[,] Apart from biochemical events, changes in cellular rheology is also a hallmark of cancer metastasis. As the formation of macrometastasis progresses, cells are exposed to a myriad of physical forces including hydrostatic pressure, shear stress, compression and tension forces.

[] In an *in vitro* 2D culture it is almost impossible to reciprocate a multitude of biochemical and biophysical aspects. Therein lies the importance of *in vitro* 3D models, which provide researchers with a wider horizon. Cancer cells can be cultured alone or in conjunction with other cell types in a spatially-relevant manner, supporting cell-cell and cell-matrix interactions.[, ,] A lion's share of such studies have incorporated breast and prostate cancer cells. Apart from them, myeloma, lung, renal, colorectal and ovarian cancer cells have also been investigated in mono or multi cultures with TME components.[]

Naturally-derived materials, which are commonly utilized in hydrogel fabrication, influence cancer cell adhesion and their metastatic potential. HA is a preferred substance for bone marrow scaffold formations due to its mechanical properties.[] Pan et al. performed a comparative study between 2D and 3D culture systems using the renal carcinoma cell line 786-O in thiol-modified HA and PEG-based scaffolds. Compared to the 2D cultures, there was a strong resemblance between the gene expression patterns of the cells in 3D culture to those observed *in vivo*. Adhesion molecules, osteolytic and angiogenic factors, associated with bone metastasis, were strongly expressed in 3D rather than 2D.[] Silk has proven to be a promising material for bone metastatic models owing to its biocompatibility, strength, toughness, porosity, thermal and chemical stability.[] Kwon et al. demonstrated the 'osteomimicry' or bone cell-like behavior of metastatic prostate cancer cell line PC-3 when cultured on silk scaffolds. They displayed migration, invasion, survival and proliferation similar to their *in vivo* environment.[] Similar observations were made by Cox and colleagues, by conducting experiments with breast cancer cells in collagen-GAG scaffolds.[] Fitzgerald et al. prepared scaffolds using nanoHAP and collagen and cocultured prostate cancer cells (PC-3 and LNCaP) with OBs. MMP9, a classic marker for prostate cancer invasiveness, was enhanced in comparison to the 2D counterparts.[] Synthetic polymer-based scaffolds are also reliable tools for mechanistic understanding of bone metastasis. In 2010, Sieh et al. fabricated tissue-engineered bone constructs using medical grade PCL and TCP and then seeding them with human OBs isolated from bone tissue. In Fig.3(c) we can see that after an incubation period of three weeks in osteogenic medium, a mineralized sheet of OBs was formed and the scaffolds were encapsulated in it. Prostate cancer cells (PC-3 and LNCaP) were seeded on these constructs and an augmentation of MMP2 and 9 was noted in the cocultures. Prostate specific antigen, which is also a hallmark of prostate cancer progression, was significantly upregulated in the cocultures. Interestingly, all of these markers were absent in the monocultures without OBs.[]

Cancer cell dormancy is also an important issue that needs addressing. Marlow and colleagues devised coculture systems involving different metastatic breast cancer cell lines (MDA-MB-231, MDA-MB-453, SUM159, MCF7, BT474, ZR75-1, T47D) with bone marrow-derived MSCs in a 3D collagen biomatrix. The cellular compositions mimicked supportive and inhibitory niches. They proved that the cellular components of the TME play a pivotal role both in inducing dormancy and in re-entering the cell cycle.[]

Since bioreactors and microfluidic systems provide an opportunity to implement parameters like vascularization, several research groups have used them to establish the bone marrow microenvironment and recreate naturally occurring forces in the TME.[, ,] Dhurjati and coworkers have used a bioreactor to recapitulate the cancer-stromal cell interactions in a

bone marrow microenvironment by coculturing osteoblastic tissue with the highly invasive breast cancer cell line MDA-MB-231. A 3D osteoblastic tissue was grown from isolated pre-osteoblasts for up to 5 months in a specialized bioreactor during which active bone formation was observed in the form of OBs maturation to osteocytes. In the coculture, the cancer cells penetrated the mature osteoblastic tissue (lower cell-ECM ratio and mainly osteocyte phenotype) by ECM degradation. Downregulation of collagen and osteocalcin synthesis and upregulation of pro-inflammatory IL-6 cytokine proved disruption of osteoblastic bone formation.[] In further studies by Krishnan et al. osteoclasts were incorporated in the coculture, effectively recreating the active bone remodeling process. Degradation of collagen-rich matrix confirmed active osteoclast resorption and addition of the breast cancer cells further aggravated the process. They underwent chemotaxis towards the active remodeling site and formed colonies comprising osteoclasts, cancer cells and pre-osteoclasts. This tri-culture model could effectively mimic the 'vicious cycle' of bone metastasis and can be considered an interesting tool to evaluate efficiency of therapeutic drugs in skeletal metastasis.[]

By exercising meticulous spatial and temporal control over the gradients of soluble factors and cell-cell contacts, microfluidic systems can be used to replicate the physiological signals in a TME and hence be used to observe tumor invasion, progression and neovascularization. [] A 3D microfluidic model developed by Bersini et al. recreated a vascularized bone microenvironment with a triple culture using MDA-MB-231 cells, osteogenic-differentiated human bone marrow-derived MSCs and HUVECs. It was revealed that extravasation was significantly higher in MSC-conditioned medium and within 5 days the extravasated cells proliferated to form micrometastases ranging between 4-60 cells. Bone-secreted C-X-C motif chemokine 5 and breast cancer cell C-X-C motif chemokine receptor 2 played important roles in the extravasation process.[]

In the recent past, scores of works have been published which integrate combinations of cancer and supportive cell types in a variety of 3D *in vitro* bone marrow models. Indeed, these models have unfolded several avenues in the complex molecular mechanisms involved in the bone metastasis and helped in making headway in therapeutic studies. They have also averted the time and resource-consuming *in vivo* models and unsuccessful clinical trials. However, an efficient, reliable and cost-effective tool is yet to be commercially available.

3D printing of bone and bone marrow tissues

3D printing emerged as an innovative solution to address many technical challenges that scientists have been facing in various fields of science, engineering and medicine.[] This technique is promising in the field of biofabrication as it allows precise control over placement of different cell types and biomaterials.[] Therefore, 3D printing has attracted scientists who are working in the field of tissue engineering as it could facilitate the preparation of novel 3D scaffolds which could recapitulate the hierarchical complexity of different types of biological tissues, which is not achievable by following the conventional methods of 3D scaffold preparation. Additionally, 3D printing potentially allows not only to construct hierarchical 3D hybrid scaffolds with specific physical and biological characteristics using heterogeneous materials and biological cells, but also to personalize the

final morphological shape based on a scanned image obtained using computed tomography (CT) of a patient which would revolutionize treatment processes.[]

Producing such types of sophisticated scaffolds and models is, in particular, imperative in the field of bone tissue engineering in order to: i) fabricate vascularized artificial grafts, which can easily integrate in the human body, for the treatment of jaw bone defects as well as zygomatic bone fractures,[,] ii) build *in vitro* models to develop and test new treatment strategies for bone disease, iii) get a deeper understanding of the emergence and evolution of various types of hematological and bone-related diseases and iv) be used as a platform for expanding HSCs for transplantation. The term “3D bioprinting” is usually used to describe the process of patterning and assembling living and non-living materials to produce bioengineered structures.[] In the following, we use the term 3D printing to describe the printing of non-living materials and 3D bioprinting if living components such as cells are used. Almost all known 3D printing techniques have been used to prepare 3D bone scaffolds. including inkjet bioprinting,[] laser-assisted bioprinting, [] laser-aided gelling (LAG),[] 3D printing with a heatable nozzle,[] fused deposition modelling (FDM),[] extrusion,[] stereolithography (SLA),[] and laser sintering.[] Table 1 shows a comparison between different printing/bioprinting techniques that have been used in this field.

A broad spectra of natural and/or synthetic biomaterials in form of either bioceramics, composites, i.e. a mixture of bioceramics and polymers, or polymers have been used as bioinks for 3D printing of bone tissue.[] This wide diversity of biomaterials and composites that have been used in 3D printing of bone scaffolds or grafts allowed scientists to tune the final biomechanical and biophysical properties of the constructed scaffolds or grafts beside their biodegradability, biocompatibility and osteogenic ability that allow differentiation of MSCs to OBs.

Nevertheless, in vast majority of these scientific efforts, a simple approach to mimic the bone tissue has been used which was based on 3D bioprinting of several layers with well-defined thickness. Each of these layers was composed of parallel lines that were vertically aligned to the lines of the next and previous layers to yield interconnected porous scaffolds with pore diameters that were mainly determined through the spaces between the printed parallel lines of each layer. Presence of interconnected pores is critical for cell survival as it allows the diffusion of nutrients as well as oxygen into the printed scaffolds and could allow the vascularization process.[] These simple models, however, could not fulfill some essential requirements that are needed to develop reliable bone or bone marrow models as well as bone grafts that could mimic the hierarchical, biophysical and biological complexity of native bone tissue. This is mainly attributed to some technical hurdles of current 3D bioprinting techniques, as they do not allow simple, simultaneous and regional deposition of several different biomaterials, composites, growth factors, stem-, progenitor- and differentiated cells with high resolution and within an acceptable printing speed.[,] Some elegant strategies, however, have been made recently in this direction and a few of them are highlighted here. Atala and his research team developed a multidispensing printing module, named “integrated tissue-organ printer” (ITOP), which allowed simultaneous printing of various cells and polymers through a sophisticated printing nozzle with high printing resolution.[] The ITOP system was equipped with multiple cartridges to facilitate the

printing of multiple cell-laden composite hydrogels beside the supporting polymer (PCL) and sacrificial pluronic F-127 hydrogel. This allowed the formation of microchannels in the scaffold structure which could facilitate the diffusion of nutrients and oxygen into the printed tissue as it is shown in Fig. 4(a). A human-scale mandible bone construct, seeded with human amniotic fluid derived stem cells, could be fabricated, using ITOP technique, with a geometric structure designed based on the CT scan of a human mandible defect after traumatic injury which can be seen in Fig. 4(b). In fact, such combination of ITOP technique and CT scan of bone tissue could open the door towards reliable models of bone marrow. Cui et al. presented an integrated approach to fabricate a hierarchically-vascularized bone construct with biphasic structure by utilization of a dual 3D printing/bioprinting system composed of FDM and SLA printing techniques.[1] The fabricated construct had a geometric structure and biological function mimicking the osteon or Haversian system of natural bone as it is depicted in Fig. 4(c). The stiff regions of the constructed scaffold were fabricated using biodegradable PLA fibers printed with an FDM printer, which resulted in cylindrically shaped constructs containing a series of interconnected vertical and horizontal vascular channels. This was followed by coating the surface of the construct with dopamine as a prelude to immobilize BMP2, to facilitate the osteogenic differentiation, via mussel-inspired chemistry. Afterwards, human MSCs were seeded into the scaffold. Next, the elastic vascular structure was bioprinted using SLA printer, which could fill the interconnected channels with gelatin methacrylate (GelMA) hydrogels containing human MSCs and HUVECs. Vascular endothelial growth factor (VEGF) peptide was attached to the GelMA during the printing process via “thiol-ene” click reaction. Interestingly, the constructed scaffold showed disparate mechanical properties based on region, compressive modulus of about 0.38 GPa in the stiff parts of the construct whereas in the vascular region the elastic modulus was in the range of 10 to 30 kPa, which mimics native bone tissue. This strategy of patterning two adjacent regions with different mechanical and biological properties and coating with different growth factors could support one day the current efforts to fabricate a reliable bone marrow model since, as it was mentioned in the introduction, a native bone marrow owns several niches. Another advanced technological approach that might be used also in the future to support the scientific efforts in this field was recently reported by Khademhosseini and his research team who developed an extrusion 3D printer, which is shown in Fig 4(d) and 4(e) that was able to achieve fast and continuous extrusion of several materials, i.e. bioinks, with different properties through one nozzle.[2]

Despite the great potential of 3D printing in the field of biofabrication, this technique has not been used yet in the development of a reliable model of bone marrow tissue that recapitulates the exact anatomy and physiology of the different niches in bone marrow, but rather to construct simple models of porous scaffolds with well-controlled pore size and pattern. Zhou et al., for example, used a table top SLA 3D printer to fabricate several 3D highly porous and interconnected scaffolds with different geometric patterns.[3] PEGDA containing RGD and/or nanocrystalline HAP were used as bioinks to fabricate these scaffolds. The authors investigated the effects of pore sizes, geometric patterning and presence of low intensity pulsed ultrasound on the adhesion, the growth and osteogenic differentiation of MSCs. Braham et al. fabricated an advanced model of 3D bone marrow in order to study the expansion and interaction of primary myeloma cells.[4] The model was

composed of two separate but interacting niches, i.e. the endosteal niche, prepared by using a bioprintable paste of calcium phosphate cement with seeded osteogenic multipotent MSCs and the perivascular niche, which was prepared using Matrigel™ containing endothelial progenitor cells and MSCs. This was the first time that both – the endosteal and perivascular components of HSC niches – were included in one mold, of which one part, the endosteal one, was 3D printed.

However, as it was mentioned above, even by 3D printing, obtaining an ideal bone marrow model is not straightforward, but rather faces several critical technical challenges regarding the currently available 3D printing techniques as well as the utilized bioinks. Regarding the technical aspect, developments should be made in order to increase the speed of printing and to facilitate precise and simultaneous bioprinting of several types of bioinks in a way that they would not mix with each other during or after the printing process and does not cause damage to the printed cells. In order to achieve this goal, the development of 3D bioprinters which are able to use two or more printing techniques within one printer, and thereby facilitating printing of several biomaterials of different nature using one 3D printer, is advantageous. Furthermore, new functional bioinks should be developed to allow high speed printing using biocompatible materials with acceptable rate of degradation and immobilization of different types of growth factors.

Outlook

Utilization of 3D printing to construct a simple bone scaffold has shown some successes in the area of bone graft fabrication, but so far this was not the case for the models aimed to be used as a platform for expanding stem cells, or as *in vitro* models to test new treatment strategies or to study the different factors affecting the occurrence of bone malignant diseases. Therefore, developing a reliable model that can precisely mimic the different niches, appears to be mandatory [Fig.2 (b, VI)]. In order to reach this goal, several interrelated parameters which should be taken into consideration are: i) chemical and biochemical conditions, e.g. gradient concentration of calcium ions and sustained release of growth factors, ii) cellular composition, iii) binding sites for cell attachment, iv) nanotopography and –patterning, v) stiffness of the different niches, vi) 3D architecture, vii) tailorable degradation rates, viii) suitable supply of nutrient and oxygen, and ix) vascularization of the system. Reaching this aim will enable scientists, for instance, to construct reliable leukemic models which are able to support the diffusion of soluble factors, resembling the *in vivo* gradient. It should allow also cell migration along the gradient and provide an architecture with enough space to analyse cell-cell contacts in *in vitro* cocultures. Such a constructed model facilitates the investigation of the anchorage of LCs in the leukemic bone marrow in consequence of other cells or soluble factors in their environment. A large number of studies have been dedicated to the biochemical pathways associated with metastatic progression, but the associated biophysical cues have been highly underrepresented. These parameters are equally important in tumor pathophysiology and investigating them could open doors for new therapeutic strategies.

Besides enabling fundamental research in the context of human healthy and diseased bone marrow, bone marrow models undeniably serve an array of applications, such as *in vitro*

HSC expansion for therapeutic treatment of hematological malignancies and as tools for drug-testing. Preparing such models using 3D culture techniques, has opened up new vistas to replicate the complexities present in an *in vivo* microenvironment. As discussed earlier, these systems exert a significant advantage not only over conventional 2D culture systems but also on *in vivo* models. They facilitate co-localization of different cell types, equip perfusion of biochemical factors and provide a mechanically stable ECM in 3D, all the while circumventing the investment of resource and time necessary for *in vivo* studies. They have also imparted valuable insights into the multifaceted nature of cancer biology and aided studies on leukemic niches and tumor dissemination to bone. Over the past years, numerous bone marrow models have been researched and several strategies have been developed. However, there still remains an urgent need for cost-effective and time saving applications. Nevertheless, in case of research, systems incorporating as many characteristics as possible, should be devised. The principal idea which should be kept in mind while developing such models is 'as complex as necessary but as simple as possible'. 3D printing is a technology that stands out in this regard. It has the potential to mimic the healthy and diseased bone marrow, in varying degrees of complexities, tailored to the prerequisites of each individual study. Having said that, this technique also faces several challenges and further progress is necessary for successful practical applications.

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Fig. 1. Schematic drawing of the bone marrow niche *in vivo*.

The simplified representation of the niche in the bone marrow shows the microenvironment of HSCs (light red) consisting of several types of niche cells such as the OBs (blue) located at the endosteum, perivascular endothelial cells (red) or MSCs (purple) and biochemical stimuli due to the expression of soluble factors (red and yellow dots) by niche cells.

Furthermore, biophysical parameters (different stiffnesses reviewed in Nelson & Roy[]) throughout the bone marrow which have an impact on cells behavior are illustrated in green.

Fig. 2. Schematic drawing of the development of 3D models.

Evolution of conventional HSPC cultures into sophisticated 3D biomimetic bone marrow niches. In conventional culture systems HSPCs are cultivated and expanded on 2D polystyrene devices in combination with cytokines and small molecules (i) or in addition with feeder cells (ii). By functionalizing the substrate and varying its physical parameters such as stiffness, ECM properties were mimicked in research applications (iii). Allowing cells to grow in a 3D environment, e.g. as spheroids, embedded in a polymer matrix or in the cavities of pores of polymer scaffolds (iv), reformed culturing processes of HSPCs. Using bioreactors or microfluidic devices further improved nutrient supply and added more parameters of the niche to the culture system (v). In future research, more attempts should be made to mimic the niche in more detail, combining various substrates (shown in light green and light orange), stiffnesses (displayed as brightness of the substrate-colors), cells (depicted by the blue and the purple cell), soluble factors (presented by yellow and red dots) and a vascular system (shown as a red circle) in one model (vi).

Fig. 3. *In vitro* models of the bone marrow in health and disease.

(a) Example of a bone marrow niche model in healthy conditions: Pseudo colored scanning electron micrograph of a porous PEGDA-RGD hydrogel scaffold (blue) from Raic et al. The 3D culture system was seeded with MSCs (purple) from the bone marrow and HSPCs (red). Arrows indicate the size of the cells (MSC $\approx 28 \mu\text{m}$, HSPC $\approx 12 \mu\text{m}$). Scale bar = $20 \mu\text{m}$. Adapted/reproduced with permission from Elsevier, ref.[] (b) *In vitro* model of a leukemic niche: Microvessel system lined by endothelial cells mimicking the vascular niche developed by Kotha and colleagues. The LCs (blue) were perfused in the microvessel system which was embedded in collagen type I (left). Addition of fibroblasts (HS5-cells; green) into the matrix around the vessels led to increased adherence and migration of LCs (right). Scale bars = $100 \mu\text{m}$. Adapted/reproduced with permission from BioMed Central, ref.[] (c) A model simulating prostate cancer bone metastasis: On the left is a scanning electron microscopy image of the scaffold prepared from mPCL-TCP and then seeded with human OBs. Within 3 weeks, OBs formed a sheet on the scaffold. Scale bar = 2 mm . On the right is the immunofluorescent staining of OBs with phalloidin (red) and PC-3 cells with anti-pan CK (green) after 6 days of coculture. PC-3 cells are characterized by a spherical (white open arrow) and fibroblast appearance (white closed arrow). Scale bar = $75 \mu\text{m}$. Adapted/reproduced with permission from Taylor and Francis Ltd. ref. []

Fig. 4. Schematic drawings of some advanced 3D printing techniques for biofabrication.

(a) Illustration of the basic 3D architecture which could be achieved by using the ITOP technique to print multiple-cell hydrogels and supporting PCL polymers. (b) The upper left represents a mandible bone reconstruction designed based on a human CT image of a mandible bony defect, the upper right image shows a photograph of the 3D printed construct after culturing it in osteogenic medium for 28 days, and the lower image depicts osteogenic differentiation of stem cells in the printed construct confirmed by Alizarin Red S staining. Adapted/reproduced with permission from Springer Nature, ref. [1]. (c) Schematic illustration of designing and 3D printing of a biomimetic vascularized bone scaffold, the upper image shows the computer modeling of vascularized bone, the middle images depicts the FDM/SLA 3D bioprinting platform, while the lower image represents the microstructural design of vascularized construct. Adapted/reproduced with permission from John Wiley and Sons, ref. [2]. (d) Schematics illustrating the design of 3D printer with seven-channel printhead which is in turn connected to seven cartridges that are individually regulated by programmable pneumatic valves. (e) Side view of one end of a printed microfiber showing its internal 3D structure consisting of seven individually segmented bioinks. Adapted/reproduced with permission from John Wiley and Sons, ref. [3].

Table 1

Comparison of 3D printing/bioprinting techniques

Technique	Types	Back-ground	Advantages	Disadvantages	Ref
Inkjet bioprinting	<ul style="list-style-type: none"> • Thermal • Acoustic 	Based on ejecting droplets of liquid bioinks either by application of electrical heat or by application of voltage to piezoelectric crystals	<ul style="list-style-type: none"> • High printing speed (1- 10 000 droplets per second) • High cell viability (> 85%) • Low costs • Low preparation time • Biological compatibility with wide array of biological materials • Allow potentially printing of concentration gradients of cells, materials or growth factors 	<ul style="list-style-type: none"> • Nozzle clogging when bioinks contain fibres or high cell densities • Non-uniformity of droplet size • Possible cross contamination when printing multiple bioinks simultaneously • Limited to liquid bioinks 	[,]
Microextrusion printing/bioprinting	<ul style="list-style-type: none"> • Heatable nozzle • Low temperature extrusion using pneumatic or mechanical (piston or screw) dispensing systems 	Based on extrusion of moderately to highly viscous biomaterials or bioinks through a tiny nozzle to form filaments	<ul style="list-style-type: none"> • Compatible with a wide range of biomaterials' viscosities • Facilitates bioprinting of vascularized constructs • Low cost • Low to medium preparation time • Allow bioprinting of scaffold-free bioinks 	<ul style="list-style-type: none"> • Low printing resolution hinders fabrication of constructs with vascular microchannels • Intermediate cell viability due to cell damage caused by shear stress when using highly viscous bioinks, small nozzle diameters and large dispensing pressure. 	[,]
Laser based printing/bioprinting	<ul style="list-style-type: none"> • Laser-assisted bioprinting (LA) • Selective laser sintering (SLS) • Laser-aided gelling 	Focusing pulses of laser beam onto an absorbing substrate to generate pressure that ejects the bioink layer (LA) or onto a thin layer of biomaterial (SLS)	<ul style="list-style-type: none"> • High cell viability due to absence of nozzle • High printing resolution • Facilitated construction of vascularized scaffold 	<ul style="list-style-type: none"> • High costs • Long preparation time • Inability to 3D print heterocellular constructs • Limited commercial availability 	[,]