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REVIEW ARTICLE

Biomimetic 3D bioprinting approaches to engineer the tumor microenvironment

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Abstract

With the increasing incidence and mortality rates, cancer remains a major health challenge in the world. Despite advances in therapies and clinical programs, the efficacy of anti-cancer drugs often fails to translate from pre-clinical models to patient clinical trials. To date, pre-clinical cancer models, including two-dimensional cell cultures and animal models, have limited versatility and accuracy in recapitulating the complexity of human cancer. To address these limitations, a growing focus has fostered the development of three-dimensional (3D) tumor models that closely resemble the *in vivo* tumor microenvironment and heterogeneity. Recent efforts have leveraged bioengineering technologies, such as biofabrication, to engineer new platforms that mimic healthy and diseased organs, aiming to overcome the shortcomings of conventional models, such as for musculoskeletal tissues. Notably, 3D bioprinting has emerged as a powerful tool in cancer research, offering precise control over cell and biomaterial deposition to fabricate architecturally complex and reproducible functional models. The following review underscores the urgent need for more accurate and relevant 3D tumor models, highlighting the advantages of the use of biofabrication approaches to engineer new biomimetics platforms. We provide an updated discussion on the role of bioengineering technologies in cancer research and modeling with particular focus on 3D bioprinting platforms, as well as a close view on biomaterial inks and 3D bioprinting technologies employed in cancer modeling. Further insights into the 3D bioprinting tissue-specific modeling panorama are presented in this paper, offering a comprehensive overview of the new possibilities for cancer study and drug discovery.

Keywords: Cancer modeling; 3D bioprinting; Biomimetic; Disease modeling

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1. Introduction

Cancer is a chronic multi-factorial disease and is still among the major leading causes of death worldwide[1,2]. Cancer incidence and mortality are rapidly growing. To date, 1 in 8 men and 1 in 10 women are anticipated to be diagnosed with cancer in their lifetime[3]. Despite recent advancements in the development of new therapies and clinical initiatives, cancer progression can hardly be slowed down, posing a major challenge to the global health care system. In addition, the majority of anti-cancer drugs that resulted in tumor regression during pre-clinical testing were ultimately found to be ineffective in clinical trials.

Thus, the standard pre-clinical cancer models have limited versatility and accuracy, which are inadequate to recapitulate complex biological diseases, such as cancer[4]. Safe and effective pre-clinical cancer models are needed not only for drug screening but also as a tool for a better understanding of cancer growth and metastasis mechanism. Conventional cancer models, such as two-dimensional (2D) cultured cancer cell lines and animal models, can poorly recapitulate the patient-specific cancerous tissue, invalidating drug testing and drastically limiting further development.

The awareness of these limitations has resulted in the recent ongoing efforts toward the development of three-dimensional (3D) tumor models[5,6]. To date, research is focusing on designing physiologically relevant 3D models that are able to closely resemble the *in vivo* tumor microenvironment and heterogeneity. In light of these challenges, new bioengineering technologies have emerged in the last decade (e.g., biofabrication) and have been used to engineer platforms to mimic both healthy and diseased organs, ultimately overcoming some of the aforementioned limitations[7,8]. Particularly, 3D bioprinting technology has come to the fore for functional applications in cancer research, offering multiple strategies to precisely dispense cells and biomaterials to fabricate geometrically complex bioengineered structures with high reproducibility[9]. Importantly, *in vitro* 3D models offer a plethora of advantages and specifically the tailored design of cancer drugs following the physiological response of cancer patients, with wide application in the new field of personalized cancer medicine, such as patient-specific immunotherapy[10].

Crucially, this comprehensive review highlights the urgent need for accurate and functionally relevant 3D tumor models, showcasing the specific use of biofabrication approaches to engineer biomimetics platforms. The complexity of the mutual interactions between cancer cells and extracellular components within the tumor microenvironment is particularly discussed, highlighting key features needed for the engineering of complex cancer models. A library of biomaterial inks and 3D bioprinting technologies available for the fabrication of cancer models is listed. Ultimately, a comprehensive report of the most relevant literature contributions on 3D bioprinting of tissue-specific models is presented in this review, with particular emphasis on metastatic 3D model, to offer a thorough support for the engineering of bioinspired cancer models.

2. Tumor microenvironment

Over the last two decades, cancer research has proved that tumors cannot be identified merely as a sole agglomerate of proliferating malignant cells[11]. Hanahan and Weinberg[12] attempted to rationalize tumor complexities by describing eight distinct hallmarks of cancer as specific functional abilities acquired by cancer cells during the development of tumors. Cancerous cells should be (i) sustaining proliferative signaling, (ii) resisting cell death, (iii) evading growth suppressors, (iv) enabling replicative immortality, (v) deregulating cellular energetics and metabolism, (vi) initiating angiogenesis, (vii) activating invasion and metastasis, and (viii) avoiding immune destruction[12,13]. Thus, cancer cells can be considered the driving force of tumor growth and progression, when supported by a cooperative variety of factors[14].

Cancer agglomerates are prone to expand and attract a heterogeneous population of cells, ultimately recruited to shape (Figure 1) a tumor microenvironment (TME)[15]. The majority of the hallmarks of cancer are fostered and sustained by the contribution of stromal cells[16]. Indeed, TME is typically composed of cancer stem cells (CSCs), stromal cells (such as mesenchymal and immune cells, endothelial cells)[17-20], extracellular matrix (ECM)[21], and a plethora of cytokines and growth factors[22,23]. Cancer cells are able to direct and manipulate the function of cellular and non-cellular components through signaling networks, orchestrating events such as immunosuppression (via mechanisms including recruitment of immune suppressive cells at the tumor site, release immunosuppressive factors, and the activation of immune checkpoints [e.g., PD-L1/PD-1, CTLA-4, LAG-3, IDO1]) that can induce the apoptosis of T lymphocytes[24-26], fibroblast recruitment and their transformation into cancer-associated fibroblasts (CAFs)[27,28], and ECM remodeling[29,30], contributing to therapeutics resistance. Taken together, these events eventually result in tumor development and progression into metastatic tumors. However, recent advances in cancer therapy have made use of tumor-infiltrating lymphocytes, harnessing these powerful assets to grow them in large numbers before administering these to the patient. Drug research progresses have recently targeted tumor-

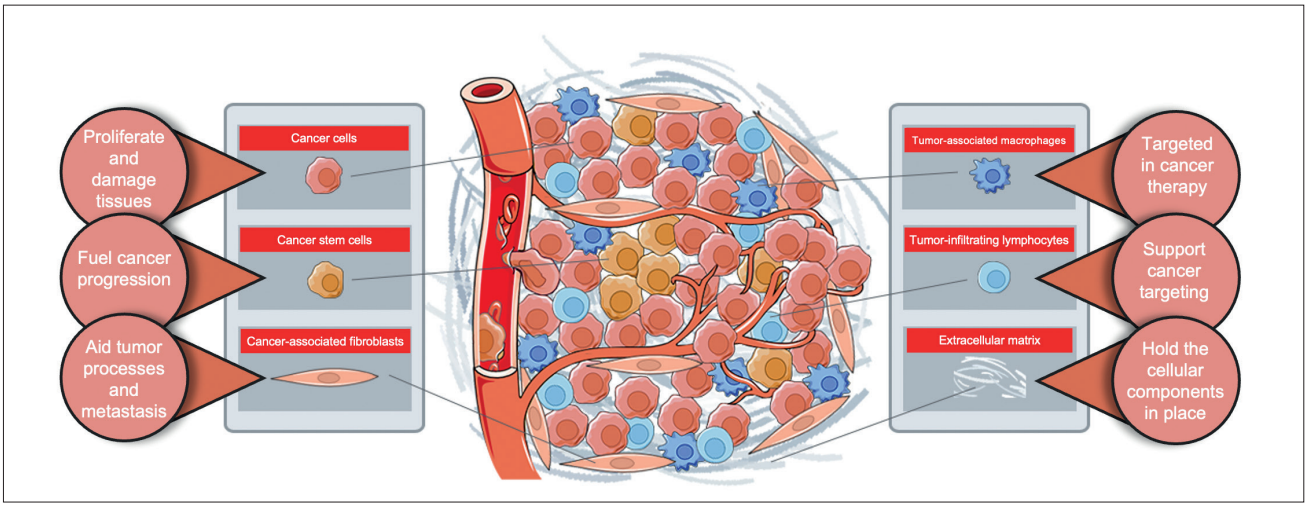

Figure 1. Tumor microenvironment (TME). The schematics summarize the principal cellular components of the TME with associated functions and the support for the maintenance, progression, and ultimate treatment of the cancerous tissue.

associated macrophages, aiming to guide phenotype polarization and thus cancer regression.

3. Engineering platforms for the study of cancer biology and metastasis

To date, most of our understanding of cancer biology is based on experiments performed either *in vitro* using conventional 2D culture models[31] or *in vivo* using xenograft animal models of human tumors[32].

2D models are economically favorable due to the low-cost maintenance of the culture and related experiments. However, 2D models are oversimplified and cannot recapitulate the native TME. In fact, 2D platforms comprise cancer cells that are cultured on flat surfaces, forced to grow as monolayers, consequently altering their morphology as well as several of their functions, critically failing to recapitulate the physiological cell–cell and cell–matrix interactions[33]. Currently, bidimensional platforms are not able to represent the tumor heterogeneity and the numerous cellular components of the TME. Moreover, unlike cancer cells *in vivo*, cells in 2D culture models receive a continuous supply of nutrients, oxygen, and other molecules which are abundantly present in the supplemented medium.

Animal models have been extensively employed for cancer research. In particular, mouse models, ranging from xenograft tumors to genetically engineered mice[34], are the most used model systems because of the low cost, small size, ease of use, and known genetic information[35]. In xenograft tumor models, cancer cells are transplanted into immunocompromised mice and allowed to grow. Although cell line-derived xenografts have the advantage to resemble the TME, cancer cells are prone to adaptations to *in vitro* growth, losing the native characteristics of the tumor. Recently, patient-derived xenografts (PDX) have been frequently used since these models are able to recapitulate the main characteristics of the host tumor[36]. On the other hand, animal models are more expensive compared to 2D models, demanding, time-consuming, and more importantly, unable to mimic the actual response of a human organism[37]. Furthermore, the high demand for test subjects needed for animal experimentation has raised ethical concerns and has led to the founding of several organizations demanding the replacement and reduction of animals in research[38]. To overcome the aforementioned limitations, 3D culture platforms have gained increasing interest by more closely mimicking the TME and providing more physiologically relevant information. Indeed, unlike cells cultured in plastic, 3D culture models are not constrained to a single layer and the additional dimensionality allows for the spatial arrangement of their surface receptors and also induces physical constraints among surrounding cells[39]. In the attempt to create 3D platforms that may recapitulate the pathophysiological functionality of TME, 3D cancer models have been developed, ranging from spheroids cultures to biomaterial scaffolds and tumor-on-a-chip platforms.

Multi-cellular tumor spheroids are a scaffold-free 3D cancer cell-only platform typically consisting of multiple type of cell aggregates that favor cell–cell interactions and produce their own ECM. As solid tumors, multi-cellular tumor spheroids (MCTS) display similar stiffness and spatial heterogeneity and also similar nutrients, oxygen, and cell proliferation gradients[40,41]. Even though more advanced compared to 2D culture models, a limiting factor of MCTS is the lack of the actual range of ECM

components of the TME and other relevant features, such as the supporting vasculature and the fluid dynamics.

Lastly, to overcome the disadvantages of MCTS models, tumor-on-a-chip[42] platforms have emerged as microfluidics cell culture devices designed to recapitulate the tumor physiology by mimicking a dynamic TME, including and providing fluid flow, perfusion, and chemical gradients. Comprehensive reviews on 3D-printed tumor-on-a-chip have been recently released with detailed insights and information[43,44].

However, although these approaches may serve as useful tools to understand the roles of biochemical and physical cues in tumor initiation and progression, these strategies lack the ability to control the location and organization of multiple cells in a complex system such as the TME.

In the last decade, tremendous efforts and progresses have been made in the development of 3D culture models that can more accurately resemble the *in vivo* tumor milieu. To this purpose, 3D bioprinting technologies and advanced biomaterials are gaining more interest because of the potential to form more complex and well-organized constructs and to better control the distribution of the cells within the 3D structure[45]. Moreover, 3D bioprinting relies on the capability of building a full range of large-scale tumor models with multiple biomaterials, various cell types, and perfusable networks with high resolution and reproducibility[46] (Figure 2).

3.1. Biomaterial inks

The biomaterials used to engineer a 3D cancer model should be selected to resemble the native TME, providing cells not only with a scaffolding structure, but also with appropriate biochemical, mechanical, and physical cues. Therefore, it is of utmost importance that the specific biomaterial mimics the physiochemical characteristics of the native ECM[17,47]. Particularly, for 3D bioprinting applications, biomaterials which can be adopted for use as biomaterial inks[48] are properly named bioinks following the addition of living cells[49,50]. Specifically, when designing a biomaterial ink, the mechanical and biochemical properties of the ink are needed to be taken into consideration for the printability and the biocompatibility of the constructs[51]. Therefore, the limitations imparted by the material itself and the choice of the bioprinting technology inevitably narrow the range of biomaterials available to engineer a 3D-bioprinted cancer model. Typically, biocompatible hydrogel material inks (>90% w/v water) can be synthesized from a wide array of naturally derived and synthetic polymers.

Naturally derived polymers are obtained from natural sources and can form hydrogels that usually demonstrate good biocompatibility and biodegradability. Naturally derived polymers could be further classified based on the native source. Indeed, polymers such as alginate, agarose, or gellan gum are obtained from plant-based or living organisms like algae or seaweeds and lack specific motifs for cell adhesion, whereas others, such as collagen, gelatin, fibrin, and even decellularized tissue-specific ECM materials, are derived from xenogeneic sources (generally vertebrates), which exhibit the inherent ability to foster cell adhesion. Despite the elevated biocompatibility and ECM-like properties, hydrogels formed from naturally derived polymers have some limitations, such as their weak mechanical properties (compared to synthetic hydrogels) or batch-to-batch variability[52], which may lead to low reproducibility and consistency.

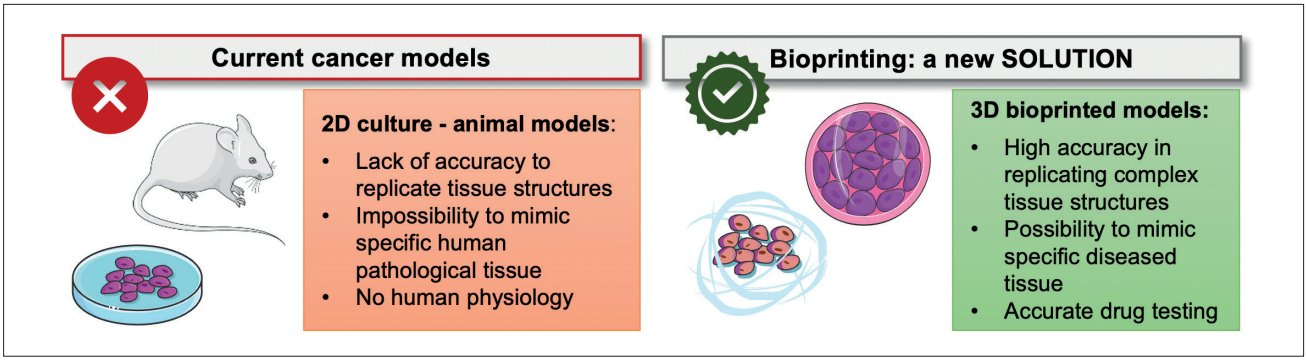

Figure 2. The evolution of cancer modeling. Standard pre-clinical cancer models often lack versatility and accuracy, making them inadequate for replicating complex biological diseases, such as cancer. Conventional cancer models, such as two-dimensional (2D) cultured cancer cell lines and animal models, struggle to accurately reproduce patient-specific cancerous tissue, compromising drug testing and significantly limiting further development. Thus, inherent physiological differences with humans, resulting in altered drug response, remain crucial considerations for the final testing of cancer therapeutics. Safe and effective pre-clinical cancer models are needed for drug screening and a better understanding of cancer growth and metastasis mechanisms. 3D bioprinting is emerging as a key technology for the rapid and reliable engineering of cancer-like tissue.

On the other hand, synthetic polymers such as polyethylene glycol (PEG) are able to form hydrogels with tunable mechanical properties, such as stiffness or degradation rate, but often lack the ability to recapitulate the native ECM due to the absence of any motif for cell adhesion. However, synthetic polymers can be engineered by chemical functionalization with various bioactive moieties, such as integrin-binding or enzymatically degradable (e.g., MMP-cleavable) peptide sequences, to enhance the ultimate functionality.

Harnessing the advantages of blending naturally derived and synthetic materials, composite inks are preferred for the fabrication of biomimetic TME models. Nevertheless, the combination of tissue type, material ink, and bioprinting technology has offered a challenging choice over the possibility of printing functional cancerous models *de novo*.

3.2. 3D bioprinting of tumor microenvironments

3D bioprinting is a revolutionizing technique in which 3D structures are fabricated via layer-by-layer deposition of biomaterials, living cells, and biomolecules for tissue engineering and regenerative medicine purposes[9]. Typically, 3D bioprinting offers several advantages, such as the ability to provide high control over spatial and temporal deposition of cells, alongside the fabrication of structures with precise size and controlled architecture. Moreover, 3D bioprinting enables the construction of tissue models in a high-throughput manner, which is indispensable to meet the need for more reliable and standardized models for anti-cancer drug screening[17].

Indeed, during the last decade, researchers have attempted to reproduce the complexity of the tumor milieu via 3D bioprinting, building biomimetic 3D *in vitro* tumor models. Taken together, the capability of using multiple cell types (including all the elements that make up the TME) and different biomaterials, along with the possibility to develop a functional vascularization, made 3D bioprinting an attractive and promising strategy to engineer 3D *in vitro* tumor models.

In the following section, the main 3D bioprinting techniques are briefly described, followed by the discussion of the more relevant studies on the development of 3D-bioprinted models that resemble various TMEs and their main features, classified by the bioprinting technique employed (Figure 3).

3.2.1. Inkjet-based bioprinting

Inkjet-based bioprinting[46] (IBB) is a non-contact 3D printing technology that allows for the positioning of cells and biomaterials into a desired pattern using small

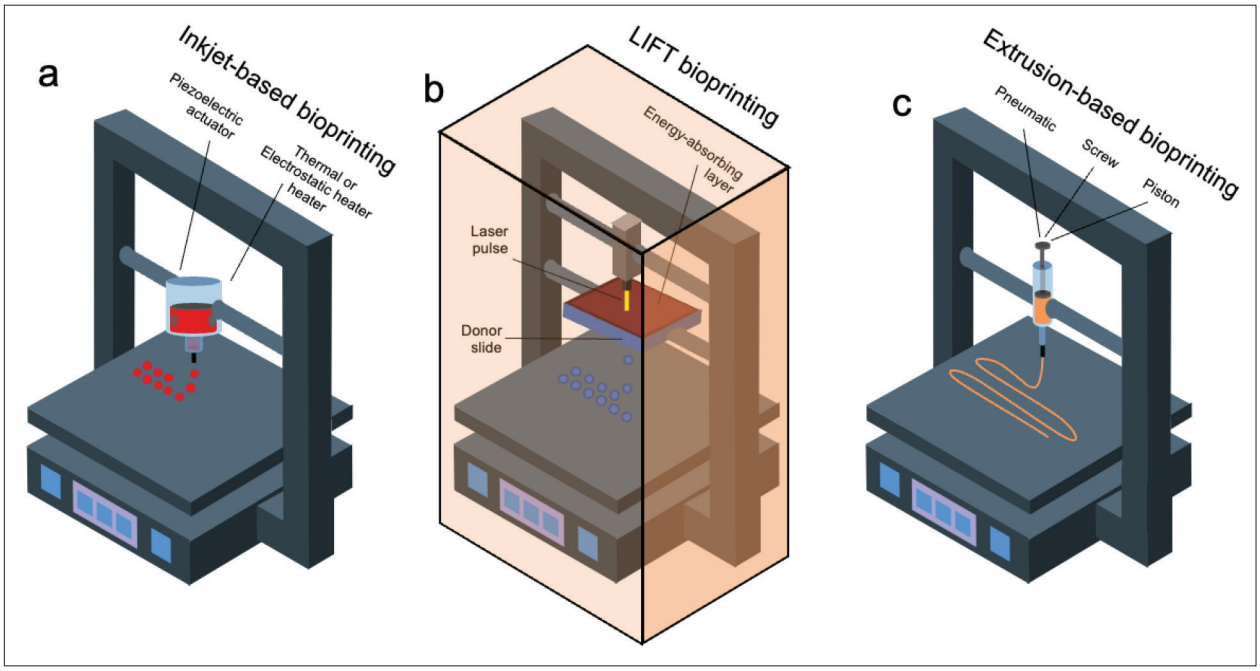

Figure 3. 3D bioprinting approaches currently explored for the fabrication of 3D cancer models. (a) Inkjet-based. Scaffolds are assembled drop-by-drop, and the fabricating ink droplet methods include piezoelectric and thermal propulsion for inkjet-based bioprinting. (b) Laser-based. This method is nozzle-free, and the scaffold is produced by transferring the cell from a donor slide to a collector using the laser. The donor site is covered by an energy-absorbing layer, and the reaction with the beam causes a precise ejection of cells. (c) Extrusion-based. This is the most widespread bioprinting approach in cancer modeling, due to the ease in control and tissue development.

volumes in the form of droplets which are dispensed by thermal or mechanical processes. IBB is a relatively low-cost technique and provides high cell viability[53] with elevated speed and high resolution[54]. IBB has been extensively employed during the last decade for the recapitulation of TMEs. Despite the numerous benefits, there are also several limitations, such as the use of polymer solutions with low concentration[9] and the lack of droplet stability and directionality during ejection. Nevertheless, IBB has been recently proven efficacious for the high-throughput patterning of cancerous micro-tissues for the rapid screening of therapeutics[55]. Overcoming the above-mentioned limitations and by harnessing the rapid planar displacement of IBB technology, cancer microenvironments could be modeled accurately by patterning tumor cells with nanoliter precision.

3.2.2. Laser-induced forward transfer bioprinting

Laser-induced forward transfer (LIFT) is a non-contact, nozzle-free technique that uses a pulsating laser as the energy source to irradiate a donor slide, causing the formation of microbubbles that propel the biomaterial onto the receiving slide in droplet form[56]. Tuning the laser energy source and printing speed[57], high-resolution 3D structures can be printed, reaching single-cell droplet accuracy[58].

In addition to the high resolution and precision of the constructs, a great advantage of laser-based technologies for 3D bioprinting applications is the absence of the nozzle that allows for fabrication with no concern for viscosity or clogging during printing. However, there are several limitations, such as the lower cellular viability[59] or the higher cost of the system, compared to other printing technologies. The exploration of the potential use of LIFT in cancer modeling has been ongoing in recent years, uncovering new ways of patterning high-throughput platforms for drug screening studies[60]. The ability of cancer tissue to resist thermal and mechanical stresses is fostering the use of laser-assisted bioprinting technologies to fabricate new biomimetic models, facilitating the engineering of new multi-cellular cancer microenvironments.

3.2.3. Extrusion-based bioprinting

Extrusion-based bioprinting (EBB) is currently the most widely used bioprinting platform in developing 3D tumor models. Biomaterial inks, used in EBB, are loaded in cartridges and extruded through a nozzle by pneumatic or mechanical forces, which allows for continuous deposition of the material in a predetermined 3D structure. Among its many advantages, EBB can rely on high printing speed and the possibility to print constructs with high cell density and good viability. Hydrogel-based materials are the most reliable inks to be used in conjunction with living cells with EBB platforms. Nevertheless, specific physicochemical parameters must be optimized prior to extrusion. For instance, hydrogels with high viscosity inevitably expose cells to high shear stress at the nozzle and also to increased pressure in the syringe during printing, both of which can greatly impact cell viability[61,62].

However, EBB is a low-cost, simple and highly flexible technology that has been adapted to house multi-material and co-extrusion printing[63,64]. Harnessing the EBB functionality, 3D *in vitro* cancer models used for investigating tumor progression and anti-cancer drug resistance have been recently fabricated. For instance, 3D matrices printed through EBB can function as ideal platforms to promote the formation of tumor spheroids and offer long-term proliferation cell culture.

Ultimately, EBB is a popular 3D bioprinting platform to develop 3D tumor models not only because of the associated low-cost and simple utilization but also by the virtue of flexibility and suitability for implementations (e.g., co-extrusion, microfluidic-assisted bioprinting[34]). On the other hand, the choice of biomaterial ink is subjected to strict requisites and determined parameters (e.g., viscosity), which could significantly impact cell viability.

However, the latest advancement of engineering technologies has greatly facilitated the customization of EBB set-up to accommodate the fabrication of functional tumor models. Thus, a variety of novel approaches, such as the direct printing of cell, spheroid, organoid printing[65], as well as the engineering of new vascularized structure[66], have been attempted lately. Typically, the low resolution (>100 μm) cannot allow to create highly detailed structure, but recent effort has been focused on functionalizing EBB-fabricated tissues with cancer spheroids to improve ultimate functionality of the model to validate and test new anti-cancer drugs[67].

4. 3D bioprinting bioinspired tumor models

Biofabrication is currently shaping cancer research, coming to the fore as a high-throughput screening platform to test the safety and efficacy of new drugs, while replicating pathophysiological processes and events *in vitro*. Therefore, the unparalleled ability of bioprinted and bioinspired models to recapitulate aggressive and deadly primary (breast and brain) and secondary (lung and bone) tumors and biological processes has been recently harnessed for the fabrication of novel biomimetic models. Here, we list the most recent work on tissue-specific bioprinted cancer models, providing a comprehensive library for 3D-bioprinted tumor tissue replicas (Figure 4) with a classification that can be found in Table 1.

Table 1. 3D-bioprinted cancer models

Cancer model	Bioprinting technique	Biomaterial ink	Cell types		Key features	Ref
Breast cancer	(Co-axial)	Modified alginate	Human breast adenocarcinoma cells Mouse macrophage cells	IC V	• Macrophages migration • Blood vessel-like structures	[71]
	Extrusion-based	Gelatin and alginate	IMR-90 fibroblast cells MDA-MB-231 breast cancer cells		• Migration of fibroblasts	[70]
	Extrusion-based		Breast cancer cells Normal mammary epithelial cells		• Chimeric organoids underwent epigenetic alterations	[72]
	Inkjet	Gelatin (as template for sacrificial arrays) PEG-DMA (for concave wells)	MCF7 human breast cancer cells	TG	• Promote spheroids formation	[68]
	Laser-based (direct write)	Gelatin, alginate and chitosan	MDA-MB-231 human breast cancer cells	TG	• Multi-cellular spheroids formation • Effect of tumor spheroids size on internalization of transferrin	[120]
Metastatic breast cancer	Laser-based (stereolithography)	GelMA + nHA	MDA-MB-231 breast cancer cells Primary human bone marrow MSCs Osteoblasts	V	• Higher VEGF expression	[69]
	Laser-based (stereolithography)	PEG-DA + PEG + nHA	MDA-MB-231 breast cancer cells Human fetal osteoblast cell line (hFOB)	TG	• Promote spheroids formation	[121]
CNS tumors	Extrusion-based	Gelatin, alginate, and fibrinogen	Glioma stem cells	V	• Higher VEGF expression Higher chemoresistance to temozolomide	[75]
	(Co-axial)	Alginate and gelatin	GSC23 human glioma stem cells Human mesenchymal stem cells (HMSCs)		• Tumor–stroma cells interaction	[122]
	(Co-axial)	Alginate	GSC23 human glioma stem cells U118 glioma cells	EV	• Higher VEGFR2 expression • Higher matrix metalloproteinases (MMPs) expression	[123]
	Extrusion-based	GelMA and gelatin	Glioblastoma-associated macrophages (GAMs) Glioblastoma cells	IC	• Interaction between glioblastoma-associated macrophages and glioblastoma cells	[76]
	Extrusion-based	RGD-alginate Hyaluronic acid Collagen-1	U87MG glioblastoma cells MM6 monocyte/macrophages		• Higher chemoresistance to cisplatin	[124]
	Extrusion-based	GelMA and algMA	SK-N-BE neuroblastoma cells	V	• Blood vessel-like structures	[77]
	Inkjet-based	Agarose and collagen	Human primary umbilical cord-derived mesenchymal stromal cells (UC-MSC) Human primary umbilical vein endothelial cells (HUVEC) Human bone marrow-derived epithelial–neuroblastoma immortalized cells (SH-SY5Y)		• Formation of Homer–Wright-like rosettes • Formation of vimentin-rich matrices	[74]
Lung cancer	Extrusion	Gelatin and alginate	NSCLC PDX cell line Lung CAFs		• Cellular crosstalk between CAFs and NSCLC cells	[81]
	Extrusion-based	Gelatin, alginate, fibrinogen	Human vascular endothelial cells (HUVECs) Lung fibroblasts (LFs)		• Cell proliferation, mRNA metabolism, and anti-apoptotic activity increased with stiffness	[80]
Bone cancer	Extrusion-based	Hydroxyapatite, poly(dopamine), carboxymethyl chitosan	Murine bone marrow stroll cells (mBMSCs)		• High application potential of composite scaffold in bone tumor therapy	[90]
	Extrusion	Alginate, gelatin, chitosan	Saos-2, UMR-106 osteosarcoma cell lines	TG	• Proof of concept for the engineering of a novel osteosarcoma ink	[94]
	Extrusion-based	Alginate, gelatine	Saos-2		• Active hydrogel for bioartificial bone tissue	[97]

Studies were classified based on the specific tissue type. Key features of reported studies have been reportedare mentioned in the table,: "highlighting Tumour growth, TG" stands for: tumor growth (formation of spheroids, long-period culture, etc.);, "IC": stands for Iimmune cells, IC; ECM, "E": stands for ECM (deposition, remodelling, degradation, etc.); , Vasculature, "V" stands for: vasculature (vessel-like structures, angiogenesis etc.); and, Metastasis, "M:" stands for metastasis.

4.1. Breast cancer

Breast cancer is among the deadliest malignancies affecting women worldwide, progressing through specific stages from epithelial hyperproliferation to metastasis. Modeling 3D breast cancer TME has proved to be effective for drug testing and emulating drug resistance mechanisms.

Ling *et al.*[68] used a custom-built bioprinting system to print sacrificial gelatin arrays as templates for fabricating concave wells and *in situ* seeding of breast cancer cells to form cellular spheroids in a controlled and high-throughput manner (Figure 4a). Similarly, Zhou *et al.*[69] employed the same method to fabricate bone matrices composed of gelatin methacryloyl (GelMA) and nano hydroxyapatite (nHA) and observed the interaction between breast cancer cells and stromal cells (hFOB cells and MSCs). Relevantly, breast cancer cells were found to inhibit cell proliferation of osteoblasts and MSCs. Vascular endothelial growth factor (VEGF) was found overexpressed and secreted by breast cancer cells with associated decreased alkaline phosphatase (ALP) activity of osteoblasts[69]. Jiang *et al.*[70] used a composite hydrogel biomaterial ink, composed of gelatin and alginate, to embed and subsequently extrude breast cancer cells and cancer-associated fibroblasts (CAFs). The bioprinted co-culture models were able to provide a biomimetic environment for more than 30 days and showed the formation of MCTS after 7 days of co-culture. Moreover, after 15 days of co-culture, fibroblasts migrated through a non-cell region of the hydrogel matrix and infiltrated the MCTS[70].

Recently, the modeling of the immune response to breast cancer progression has been modeled by Grolman *et al.*[71], who fabricated vessel-like structures by extruding peptide-conjugated alginate under controlled flow rates. The core of the fibers was filled with macrophages (RAW 264.7 mouse macrophages), while tumor cells (MDA-MB-231 human breast adenocarcinoma cells) were incorporated in the surrounding peptide-modified alginate to support cell adhesion. By changing the architecture of the fibers, these highly tunable models allowed to investigate the interactions between tumor cells and other cell types of the TME and could be useful in resembling vasculature and modeling metastasis[71].

Reid *et al.*[72] developed a platform to investigate tumorigenesis and the process of TME control of breast cancer. A 3D collagen-based model was engineered, to incorporate breast cancer cells and mammary epithelial cells to drive tumoroid and chimeric organoids formation. The TME-driven mechanism of epigenetic alterations of cancer cells within chimeric organoids was confirmed by a significant increase in 5-hydroxymethylcytosine (an intermediate of active DNA demethylation process) levels compared to tumoroids[72].

4.2. Central nervous system tumors

Glioblastoma (GBM) is the most common malignant primary brain cancer worldwide[73]. The incidence GBM is lower compared with other primary cancers, but it is particularly aggressive and impactful for the patients' quality of life. Thus, the urgency for new therapeutic treatments is recently fueling the engineering of functional GBM models.

Recently, Campos *et al.*[74] developed a 3D-bioprinted neuroblastoma model by printing human bone marrow-derived epithelial-neuroblastoma immortalized cells (SH-SY5Y), human primary umbilical cord-derived mesenchymal stromal cells (UC-MSC), and primary human umbilical vein endothelial cells (HUVECs) with a collagen type I-based biomaterial ink. Cancer cells within the bioprinted constructs showed the formation of Homer–Wright-like rosettes (phenotypic hallmark of neuroblastomas) and produced vimentin-rich matrices (characteristic of an aggressive phenotype), triggered by the presence of MSCs within the bioprinted model[74].

Dai *et al.* employed a similar ink system composed of gelatin, alginate, and fibrinogen, to embed glioma stem cells and build a 3D-bioprinted model by mimicking the brain tumor microenvironment. The 3D-bioprinted model exhibited higher resistance to temozolomide (an alkylating anti-tumor agent) compared to 2D culture models and higher expression of nestin and VEGF, showing the vascularization potential of glioma stem cells[75].

Similarly, Heinrich *et al.*[76] developed a platform to study the interaction between glioblastoma cells and macrophages. With the fabrication of bioprinted mini-brains, a highly controlled TME was engineered to recruit GBM-associated macrophages (GAM) and polarize them into a GAM-specific phenotype. Furthermore, the study demonstrated how therapeutics that inhibit the interaction between GAMs and glioblastoma cells lead to diminished tumor growth and reduced chemoresistance[76].

Recently, Monferrer *et al.*[77] used a 3D-bioprinted platform, based on malignant neuroblastic cells and hydrogels made from GelMA and different percentages of methacrylated alginate (AlgMA), to study the effects of ECM stiffness on neuroblastic cells over time. Their findings showed an increase in cell proliferation, mRNA metabolism, and anti-apoptotic activity with stiffness, while cell cluster density and occupancy decreased[77].

Furthermore, Yi *et al.*[78] used 3D bioprinting to fabricate a 3D GBM model consisting of patient-derived tumor cells, vascular endothelial cells, and decellularized ECM in order to recapitulate the main features of native GBM (Figure 4b). Using this platform, the authors observed that this model produced evidence that matched clinically

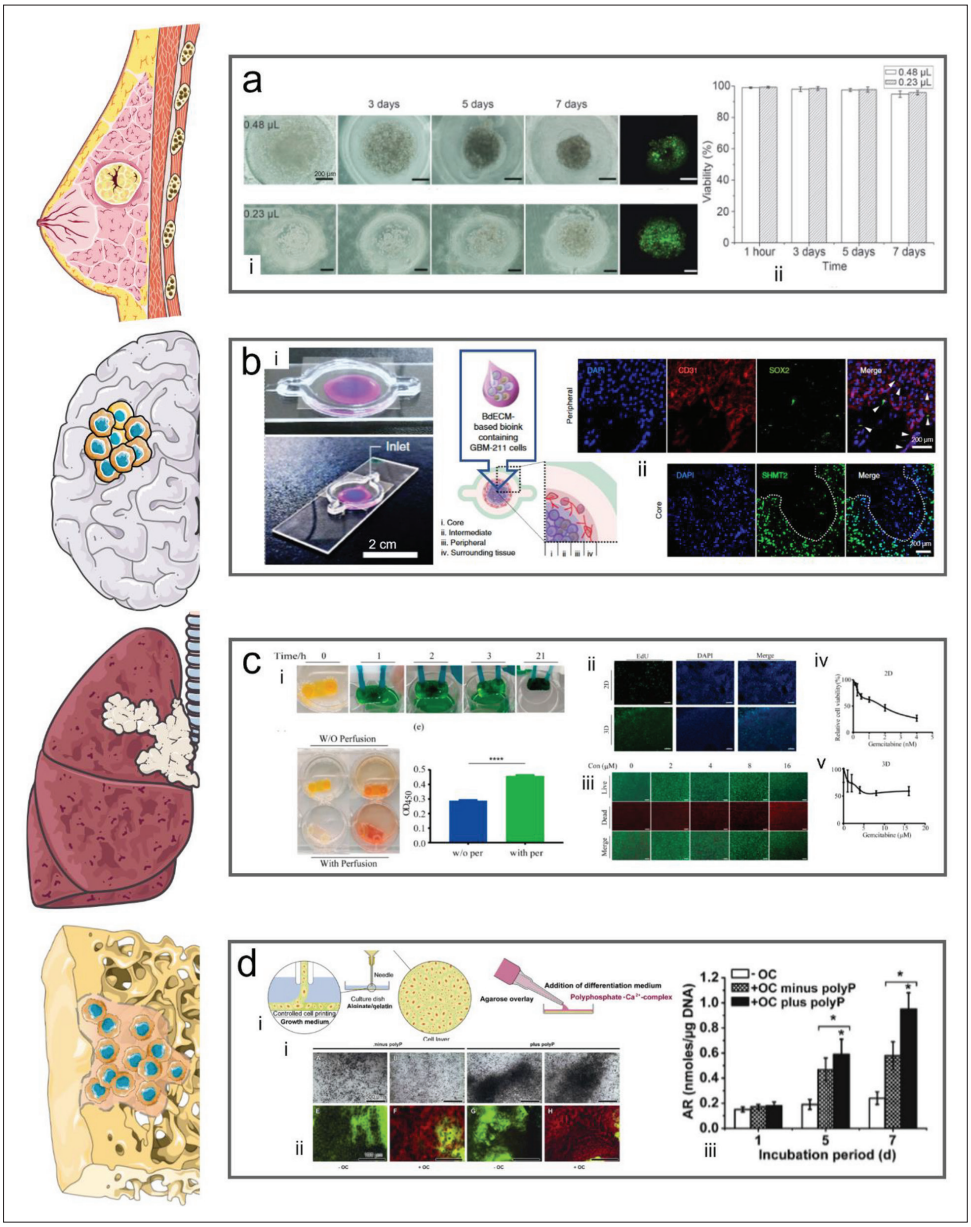

Figure 4. Tissue-specific cancer 3D-bioprinted models. (a-i) Cellular spheroids are deposited in concave hydrogel wells, and the cell development was observed for 7 days. Two sizes of droplets, 0.48 µL and 0.23 µL, were used; calcein and ethidium bromide staining were applied, and the same cell-seeding density of 1×10^6 cells/mL was used. To examine the impact of cell density on spheroid formation, the constructs were examined with live/dead fluorescence images which show the viability of encapsulated cells (ii) right after (1 h) and over long-term culture (3, 5, and 7 days). Adapted with permissions from ref.[68]. (b-i) Micrographs and a visual representation in diagram form depicting the compartmentalized structure of 3D GBM, which is composed by different various bioinks and other materials to mimic a compartmentalized structure. In the middle, there is BdECM bioink containing GBM cells (blue). (b-ii) Representative immunostaining images in the core and peripherical regions using DAPI for cell nuclei, CD31 for HUVECs, and SOX2 for resistant cancer cells. Adapted with permissions from ref.[78]. (c-i) 3D-bioprinted model perfused in dynamic flow. Models were cultured in regular media and controlled with untreated models, following XTT reagent exposure. Perfused models were compared against the constructs cultured under static conditions. (c-ii) Cell viability was investigated against 2D controls, while (c-iii) 3D culture was exposed to gemcitabine compounds with increasing concentration to demonstrate cytotoxicity following *in vitro* culture and (c-iv) quantified and compared to 2D controls. Adapted with permissions from ref.[83]. (d-i) Schematic representation of the procedure: the alginate/gelatin/Saos-2 is filled into a cartridge. The scaffold is submersed into McCoy's medium/FCS and overlayed with an agarose layer containing poly-P·Ca^{2+}-complex as a differentiation medium. (d-ii) The effect of poly-P·Ca^{2+}-complex, and in parallel, the effect of the osteogenic cocktail on the extent of mineralization, is reported herein. The cultures were incubated in the absence (minus polyP) and presence (plus polyP) of poly-P·Ca^{2+}-complex. They were observed in the absence (- OC) and presence (+ OC) of the osteogenic cocktail. (d-iii) The graft presents a quantitative assessment of the extent of mineralization using Alizarin Red S as an indicator reagent after 1, 5, and 7 days. Adapted with permissions from ref.[98].

observed patient-specific resistances after concurrent chemoradiation using temozolomide[78].

4.3. Lung cancer

Cancerous lung tissue is highly aggressive, forming fibrotic aggregates that can impede the physiological functionality of the lungs. There is an urgent need for physiological models that can resemble lung cancerous microenvironments for the rapid and efficacious testing of anti-tumor drugs. Bioprinting can aid the modeling of pathological conditions of the lung to mimic the intricate interwoven vascular and epithelial networks[79].

In this context, Han *et al.*[80] recently built a 3D-bioprinted vascularized tumor model which involved the fabrication of a blood vessel layer obtained through the printing and culturing of HUVECs and lung fibroblasts in a gelatin/alginate/fibrinogen hydrogel, followed by seeding multicellular MCTS onto the pre-formed layer. The sprouting of new blood vessels occurred in the surroundings of spheroids, driving their increase in dimensions over time. Noticeably, treatment with temozolomide (an alkylating anti-tumor agent) and sunitinib (angiogenic inhibitor) resulted to be more successful than temozolomide alone in targeting spheroids surrounded by vessel network[80].

In another study, Mondal *et al.*[81] developed a similar biomaterial ink (comprising gelatin and alginate) to embed and print non-small cell lung cancer (NSCLC) PDX cells and lung CAFs co-cultures. The bioprinted co-culture models enabled the formation of MCTS and cellular crosstalk through the upregulation of α-SMA, vimentin, and loss of E-cadherin[81].

A recent study by Dong *et al.*[82] engineered a novel droplet-based approach to 3D-bioprint lung cancer organoids in a high-throughput fashion for rapid drug testing. The alginate and hyaluronic acid-based material ink was functionalized with RGD groups, further stimulating the functionality post-printing. In comparison with 2D control, the 3D-printed organoid system demonstrated higher viability and ultimate functionality with an expression of P-CK, MUC1, and caveolin-1.

Using a digital light processing approach, Mei *et al.*[83] fabricated a new perfusable 3D lung cancer model (Figure 4c) with NSCLC cells for screening anti-cancer drug candidates and investigating the effects of gemcitabine in static and dynamic conditions. They Mei and colleagues found a significant drug-mediated effect, confirming the safety and efficacy potential of such model.

4.4 Bone cancer

Bone tumors arising in the bone or from bone-derived cells and tissues are classified as primary and those originating in other sites and metastasize to the skeleton are as secondary[84]. Both tumor types are characterized by a composite microenvironment that comprises an array of elements, including mechanical and architectural cues, signaling proteins, and interactions between the bone tumor and the stromal cells, that overall impact growth, drug sensitivity, and ultimately therapy outcome[85].

Particularly, primary bone tumors, also known as bone sarcomas, are rare malignant tumors characterized by the uncontrolled growth of cells within the bone[86]. The current standard treatment protocol is composed of the association of surgery with adjuvant and/or neoadjuvant multi-agent chemotherapy, which leads to a 5-year survival for the most common malignant bone tumors of around 70%[87]. Conventional malignant bone sarcomas include: (i) osteosarcoma (OS), localized in the metaphysis of the long bone in adolescents near the growth plate; (ii) Ewing's sarcoma, most commonly localized in the pelvis, legs, or arms of children and young adults; and (iii) chondrosarcoma, usually confined in the pelvis, legs, or arms in middle-aged and older adults, where the cancerous cells produce cartilage[88]. The majority of primary bone tumor models have been developed, attempting to recapitulate OS features and pathophysiology *in vitro*. Indeed, OS is the most common sarcoma, and it is characterized by cancerous cells producing woven bone. While mature bone is composed of sparse osteocytes, conventional OS consists of densely populated tissue with cells that exhibit osteo-, chondro-, or fibroblastic phenotype[89].

3D bioprinting has been recently applied to the treatment of OS[90,91] as well as the generation of OS models[92-94]. Multiple studies have used OS cell lines for biocompatibility evaluation and as proliferation models in lieu of OS model engineering[95]. Indeed, only a limited number of 3D-bioprinted models have been ultimately fabricated to recapitulate the complex primary bone TME *in vitro*[91,96]. In early attempts to replicate OS TME, Neufurth *et al.*[97] biofabricated a composite ink comprising alginate/gelatin mixture for encapsulating and printing Saos-2 cells. The use of a further coating prepared from agarose and calcium salt polyphosphate [polyphosphate (polyP)·Ca^{2+}-complex] was found to actively promote the proliferation[97] and, in conjunction with bioactive glass nanoparticles[98], the ability to mineralize cancer cells (Figure 4d).

4.5. Modeling vasculature in 3D-bioprinted cancer models

The presence of a functional vascular network is pivotal for the growth and survival of cancerous tissues. Typically, vascularization plays a critical role in (i) supplying oxygen,

nutrients, and signaling molecules to the tumor, as well as (ii) facilitating the removal of waste products. However, in a pathological scenario such as cancer progression, the angiogenetic process is radically upregulated and sustained to maximize the tumor survival and spreading ratio. Typically, the oxygen diffusion within a 3D-bioprinted construct is slower than its consumption[99]. Thus, the gaseous and nutrition kinetics is a limiting factor for cancer maturation *in vitro* and often fails to replicate the highly vascularized tumor mass that is present *in vivo*. To study and recapitulate complex dynamics happening as a consequence of the intense angiogenic kinetic within the tumor mass, new studies are exploring 3D bioprinting to engineer customizable vessels within the biofabricated cancer tissue. The importance of such models has been recently highlighted elsewhere[100].

The incorporation of vascular networks in 3D-bioprinted cancer models holds great promise for advancing the understanding of tumor angiogenesis and impact on tumor growth and ultimate response to therapeutic agents. Kim *et al.*[101] have recently demonstrated the possibility to engineer 3D-bioprinted cancer-vascular model by *in situ* cell printing technology. The impact of vascular supply to cancer spheroids was investigated in three dimensions, finding that the close proximity of new vessels stimulates epithelial-to-mesenchymal transition (EMT), while affecting vascular physiology by driving inflammation. The latest study from Franca *et al.*[102] revealed the supporting effect of pericytes to guide the maturation of new vessel sprouting in 3D-bioprinted models. This approach might hold the potential to closely mimic pathological angiogenesis *in vitro*. These models may provide a functional platform to investigate how the spatial arrangement and functional properties of blood vessels influence the behavior of cancer cells, the formation of metastases, and the efficacy of anti-cancer treatments[103]. Furthermore, the ability to engineer customizable vessels within the biofabricated cancer tissue opens to new avenues for drug testing and anti-angiogenic therapies.

New engineering approaches are harnessing artificial intelligence (AI) technologies to aid the development of biomimetic vascularized cancer models[104]. The use of novel AI tools can be applied to the investigation of angiogenic potential in *ex vivo* models (such as chicken chorioallantoic membrane [CAM]) to elucidate new ways of disrupting the tumor vascularization. In turn, these new ways of modeling tumor angiogenesis might be harnessed to explore the untapped potential for the study of metastasis.

4.6. Engineering models for the metastatic niche

Tumor metastasis is believed to be the main cause of cancer-related deaths, consisting of a series of complex pathways which lead to the dissemination of tumor cells from the primary tumor site to a secondary site[105]. Metastasis consists of a complex succession of cell-biological events, which can be divided into three major events, including (i) tumor cells exiting their primary sites of growth (EMT of cancerous cells, local invasion, intravasation)[106], (ii) tumor cells translocating from their primary site (survival in the circulation, arrest at a secondary site, extravasation)[107,108], and (iii) tumor cells adapting to survive in distant sites (micro-metastasis formation, metastatic colonization)[109-111].

In particular, angiogenesis and vascularization have a critical role to play in events associated with tumor growth and metastasis[112,113]. The tumor vasculature is structurally immature, leaky, chaotically organized, and poorly perfused. Blood vessel leakiness along with interstitial fibrosis and stromal fibroblast-mediated interstitial matrix contraction elevates interstitial fluid pressure (IFP) and induces hypoxic environment[114]. Metastatic models are challenging to 3D-print, due to a superior architectural complexity and culturing methodology. In a pioneering work by Lee *et al.*[115] to engineer a biomimetic metastatic model, a microfluidic bioprinting system was used to deposit GB cells to model the GBM TME via accurate deposition and use of *ad-hoc* designed biomaterial ink. Indeed, the printed GBM cells were found to be able to spontaneously assemble into spheroids post-printing and express significantly elevated levels of CD133 proteins and DCX markers, demonstrating the ability to replicate metastatic invasiveness and niche.

Secondary metastatic sites often act as a cancer reservoir, enhancing dramatically the chances of tumor-driven death. Bone is among the most common metastatic site in patients with advanced cancer. Once tumor cells reach the skeleton, the disease is generally declared incurable, and treatment is only palliative. The majority (70%) of breast, prostate, and lung carcinomas form deadly metastases in the bone tissue[116]. More than half of the patients affected by skeletal metastasis experience at least a skeletal-related events within 24 months from diagnosis[117]. Besides having a significant impact on patients, these skeletal-related events are associated with substantial costs for the health care system[118]. Therefore, new bioinspired models that can closely recapitulate the intricate metastatic process from a primary tumor site to the skeletal tissue are urgently needed. Thus, 3D bioprinting holds the potential to generate complex shapes with precise spatially defined cell distribution, to better represent both early and late events in the development and the formation of the metastasis. Recently, Meng *et al.*[119] engineered a 3D-bioprinted *in vitro* tumor model mimicking the metastatic dissemination of primary

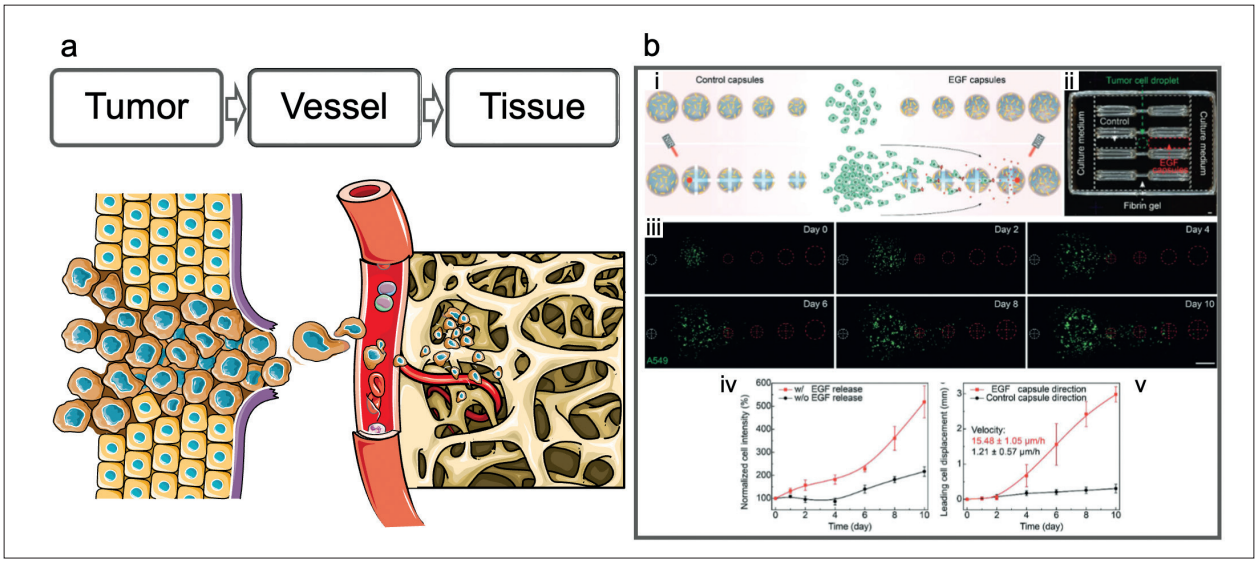

Figure 5. Engineering 3D metastatic models. (a) Schematization of metastasis niche extravasation, migration, and homing, favoring the propagation of tumor cells from the primary tumor to secondary sites. (b-i) A sketch of control and drugged capsules with epidermal growth factor (EGF) where the directional migration of tumor cells was led by EGF gradients. (b-ii) A picture of a 3D-printed culture chamber for testing the guided cell migration. (b-iii) Fluorescence images show the distribution of cells. The white circle is the control, and the red circles indicate the EGF capsules. (b-iv) The graph shows the cellular fluorescence intensity of A549 cells normalized by intensity at day 0, and EGF release (red) was compared to no EGF release (black) over time. (b-v) The plot illustrates the displacement of cells in the X-direction toward the EGF capsules, revealing the directional influence of EGF on cell migration. Adapted with permissions from ref.[119].

cancerous tissue (Figure 5). A multi-cellular TME is recreated using tumor cells and endothelial cell-lined vascular conduits within a fibrin gel containing functional fibroblasts. 3D-printed microcapsules were loaded with growth factors and selectively disrupted with a laser source to guide VEGF or EGF release. The effect of the released molecules influenced the TME progression, offering a new tool to probe the spatiotemporal evolution of specific pro-metastatic tumors.

5. Conclusion and future outlooks

In the past decade, the advances in 3D bioprinting have allowed the development of biomimetic 3D tumor models that can mimic TMEs more accurately. Despite the remarkable progresses, there are still several limitations to solve to obtain physiologically relevant *in vitro* tumor models. For instance, a high cell viability and long-term cultures, or cell native phenotypes and functions, are still difficult to maintain within 3D-bioprinted platforms. Thus, 3D biomimetic models are still far from recapitulating the complexity of TMEs. Considering these challenges, the engineering of new bioinspired material inks, along with the characteristics of the bioprinting technique and cell sources, plays a pivotal role.

Patient-derived ECM or biomaterials inks that accurately mimic the native ECM of specific tumors should be used to allow cancer cells to achieve and maintain their native phenotypes and physiological functions. Moreover, the absence of standardized bioinks in terms of polymeric composition and cell encapsulation density could lead to difficulties in the reproducibility of the experiments and in the correlation of the results. Furthermore, new features of existing bioprinting platforms, innovative implementations, and new technologies, such as microfluidic-assisted bioprinting[34], co-extrusion, or multi-material bioprinting, are promising tools to meet the need of multi-cellular and vascularized tumor models. Lastly, to overcome the use of immortalized cell lines, the use of patient-derived primary cells is promising for the development of biomimetic *in vitro* platforms for personalized drug screening and therapies. The lack of cancer-specific models is a worrisome problem, which points to the urgent need to seek the assistance of bioengineers and biologists to fabricate a model for the study of cancer progression and the test of new drugs against tumors.

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Conflict of interest

The authors declare no conflicts of interests.

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Consent for publication

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Availability of data

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