

Review

3D *In Vitro* Model (R)evolution: Unveiling
Tumor–Stroma InteractionsJoão Rodrigues,^{1,2,3} Marcel A. Heinrich,¹ Liliana Moreira Teixeira,^{4,5,6} and Jai Prakash ^{1,6,*}

The complex microenvironment in which malignant tumor cells grow is crucial for cancer progression. The physical and biochemical characteristics of this niche are involved in controlling cancer cell differentiation, proliferation, invasion, and metastasis. It is therefore essential to understand how cancer cells interact and communicate with their surrounding tissue – the so-called tumor stroma – and how this interplay regulates disease progression. To mimic the tumor microenvironment (TME), 3D *in vitro* models are widely used because they can incorporate different patient-derived tissues/cells and allow longitudinal readouts, thus permitting deeper understanding of cell interactions. These models are therefore excellent tools to bridge the gap between oversimplified 2D systems and unrepresentative animal models. We present an overview of state-of-the-art 3D models for studying tumor–stroma interactions, with a focus on understanding why the TME is a key target in cancer therapy.

Towards Biologically Relevant Tumor Models

The composition of the **tumor microenvironment (TME)**, see [Glossary](#)) and tumor stromal interactions are major factors that aggravate tumor growth and metastasis, leading to poor clinical outcomes [1–5]. There is increasing evidence that the activated stroma is a disease-defining factor, highlighting it as an important player in cancer cell invasion/extravasation, migration, angiogenesis, drug resistance [6,7], cancer stem cell maintenance [8], and immunosurveillance evasion [6,9,10].

The **tumor stroma**, the nonneoplastic part of the TME, is composed of abundant **extracellular matrix (ECM)** and multiple support cells [2], including cancer-associated fibroblasts (CAFs), endothelial cells, pericytes, immune cells [such as lymphocytes, neutrophils, dendritic cells (DCs), and monocytes] that are the most prevalent cell types ([Figure 1](#)). Other less prevalent factors include myeloid-derived suppressor cells (MDSCs) and mesenchymal stromal cells (MSCs) [7], as well as platelets [9,11]. These stromal cells actively interact with tumor cells, among themselves, and with the ECM by secreting chemokines, growth factors (GFs), enzymes, extracellular vesicles, and miRNAs that regulate the expression of genes and proteins which influence metabolic pathways related to cancer [12]. As such, some cell types can either promote or suppress tumor growth depending upon the cellular context [13]. Hence, much focus has been on accurately modeling TME interactions *in vitro* and *in vivo*.

In the field of cancer research, especially for testing anticancer drugs, many experiments are still performed with 2D cocultures, xenografts, or syngeneic mouse models. Nevertheless, 2D models are too simple and are unable to mimic the complexity and dynamic interactions of the TME. Cells grow on a flat plastic surface as a monolayer, and this can result in loss of crucial cellular signaling pathways and changes in cell responses to stimuli [14–16]. Moreover, 2D cultures do not conserve the original shape and polarization of cells ([Table 1](#)). By contrast, animal models are usually too expensive, complex, difficult to work with, and are associated with ethical

Highlights

Complex tumor–stroma interactions, a key feature of most solid tumors, drive tumor progression, metastasis, and drug resistance, and ultimately lead to treatment failure.

Clinically relevant 3D *in vitro* models are necessary to recapitulate the complex interactions between tumor and stromal cells, and provide tools to better understand the molecular mechanisms as well as for testing anticancer therapies.

Novel bioengineered models, organoid systems, and microfabrication technologies, such as 3D bioprinting, have delivered key innovations towards more advanced platforms for tumor modeling *in vitro*.

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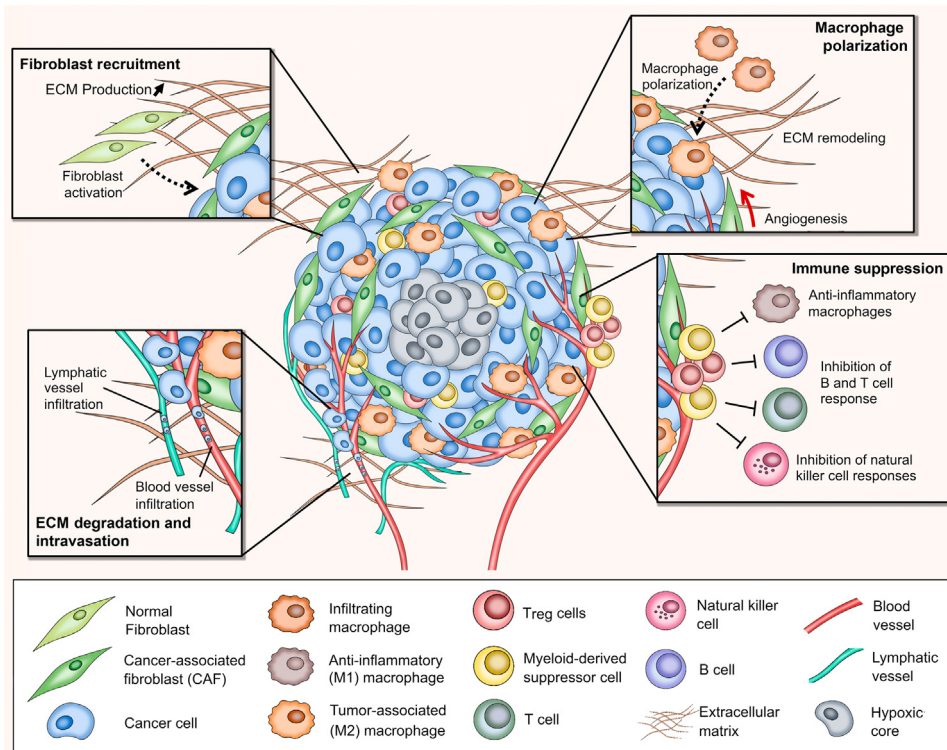


Figure 1. The Tumor Microenvironment (TME). Schematic representation of the TME describing the different cell types involved as well as highlighting key processes such as fibroblast recruitment, macrophage polarization, immune suppression, and degradation of the ECM that allows tumor cells to intravasate and metastasize. Abbreviations: ECM, extracellular matrix; Treg, regulatory T cell.

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problems. It is also challenging to analyze some effects that too often are not representative of human-specific events, which limits the applicability of these models [3,17]. By contrast, multicellular 3D *in vitro* systems can overcome these limitations and bridge the gap between experimental tractability and physiological relevance. 3D models can reproduce mechanical and biochemical cues that are crucial for cancer development, such as morphology, cell-cell/cell-ECM interactions, tissue stiffness, and specific gradients [4,14,18]. However, these models usually only feature specific interactions between one component of the TME and tumor cells. Thus, although new models are being developed, the complexity of the tumor stroma *in vitro* has not yet been achieved. Nevertheless, recent advances in 3D cancer models have the potential to (i) improve drug discovery, (ii) be used as platforms for drug testing, and (iii) enable the development of personalized cancer treatments [3].

This review presents a landscape of current 3D *in vitro* models for studying the complex interactions within the TME, including cell/ECM-based assays, cell-based models, and **microfluidics**. The advantages and limitations of each platform are discussed, followed by critical analysis of the factors that novel culture platforms should address to establish more clinically relevant 3D models.

Cell/ECM-Based Assays

ECM proteins are one of the most relevant components of the tumor stroma, and they actively interact with almost all cell types and control their behavior and responses to external stimuli.

Glossary

3D bioprinting: a subcategory of additive manufacturing – a process by which 2D cross-sections are stacked on top of one another to form a 3D patterned structure – that uses viable cells and biomaterials to produce living 3D constructs.

Decellularized ECM (dECM): ECM devoid of cellular components that maintains the native tissue architecture and composition. The process of decellularization encompasses chemical solutions (e.g., detergents, enzymatic, hypertonic, or acid/base solutions) or physical processes (e.g., stirring, sonication, high hydrostatic pressure, supercritical CO₂, or freeze-thawing).

Desmoplasia: the growth of fibrous or connective tissue that occurs in response to an external stimulus (such as an injury or neoplasia).

Epithelial-mesenchymal transition (EMT): a biological process that allows epithelial cells to adopt a mesenchymal phenotype, losing their cell polarity and cell-cell adhesions, and promoting their migration and invasion capacity.

Extracellular matrix (ECM): a 3D network of extracellular macromolecules – structural proteins (collagen, elastin), adhesive proteins (fibronectin, laminin), glycoproteins and glycosaminoglycans, and other ECM-sequestered growth factors and proteins. The interstitial connective tissue matrix surrounds all cells and gives structural support to tissues.

The basement membrane is a specialized ECM layer that separates the epithelium from the stroma.

Hydrogels: crosslinked networks of polymers that form highly porous structures that have a high degree of similarity to native ECM.

Hyperplasia: the enlargement of a tissue/organ as a result of increased cell proliferation.

Hypoxia: deprivation of adequate oxygen supply at the tissue level.

Integrins: heterodimeric cell transmembrane receptors that attach to the cytoskeleton and the ECM, and mediate cell adhesion and multiple signal transduction pathways related to cell movement, growth, differentiation, survival, and apoptosis.

Matrigel: an ECM hydrogel containing a gelatinous protein mixture derived from mouse tumor cells.

Microfluidics: the study of fluid flow in microsystems, which requires the

Dynamic changes in ECM components regulate cell proliferation, migration, adhesion, differentiation, cytoskeletal organization, and cell signaling [4,19]. For example, ECM stiffness (**desmoplasia**), alongside rapid cancer cell proliferation and the establishment of a poor blood vessel network, contributes to cellular **hypoxia** in epithelial-derived tumors [19,20]. Furthermore, secretion of hypoxia-inducible factors (HIFs) by tumor cells induces macrophage and fibroblast recruitment to hypoxic regions of the primary tumor, leading to increased ECM remodeling and angiogenesis [21].

The formation of an abnormal ECM that stimulates cancer progression starts with the activation of CAFs, which contribute to tissue fibrosis and matrix stiffness, by alignment and building up of collagen fibers (mediated primarily by LOX enzymes) [22]. In addition, deregulated ECM supports epithelial cellular transformation and **hyperplasia** [19]. Matrix metalloproteinases (MMPs) are essential for ECM degradation and tumor cell invasion, and also contribute to the formation of metastatic sites and angiogenesis support by the TME [19,22]. In addition, **integrins** are associated with every step of carcinogenesis because they function as signaling receptors that control cellular adhesion, migration, mechanotransduction, and ECM remodeling [22]. For instance, increased ECM stiffness is correlated with overexpression of $\beta 1$ integrins in cancer cells, and this induces the activation of focal adhesion kinase and RhoA/Rho-associated protein kinase signaling [22]. In addition, integrin-mediated alignment of fibronectin fibers within the tumor ECM by CAFs contributes to directional cancer cell migration [23]. These changes in the composition and architecture of the ECM over time demonstrate the complex spatiotemporal dynamics of the TME that are associated with targets of tumor progression, and can lead to promising pathways for the development of novel and more effective therapies. Given that the ECM is both a major TME component and a tumor-inducing factor, cell/ECM-based assays have been developed to manipulate the interactions between cells and the surrounding matrix in a physiologically relevant setting.

Scaffolds

An ideal scaffold should provide an appropriate environment for cell adhesion, proliferation/differentiation, and migration to allow the generation of *in vitro* tumor models that closely recapitulate essential cell–ECM interactions. In this context, tumor cells can be cultured within biomaterials, including decellularized native tissues, or in 3D scaffolds based on ceramics or synthetic and/or natural polymers. **Hydrogel**-based scaffolds are typically preferred owing to the possibility of tailoring their mechanical properties to closely mimic the tumor ECM (Figure 2).

Scaffolds produced from synthetic polymeric biomaterials include polyethylene glycol (PEG), polycaprolactone (PCL), poly(hydroxyethylmethacrylate) (PHEMA), poly(lactic-co-glycolic acid) (PLGA), and ceramics (such as hydroxyapatite or bioglass) [24,25]. Synthetic polymers allow more control over the properties of the scaffold and the ability to modulate them as required. The surface of synthetic polymers can be modified to incorporate peptides, such as RGD (Arg-Gly-Asp) peptides or fibrinogen, that promote protein adsorption and cell adhesion [5]. In addition, hybrid scaffolds combine soft hydrogels with polymeric scaffolds and cells [26]. For instance, PEG heparin hydrogels were used to demonstrate the role of integrins in tumor cell–ECM interactions by culturing breast and prostate cancer cells on hydrogels functionalized with the different peptide motifs RGD, GFOGER (collagen I), or IKVAV (laminin-111) [27]. Hence, the choice of biomaterials as well as the physical/chemical conditions of the scaffold determine how the cells will react to the substrate and play an important role in the experimental outcome (Figure 2A,B).

Natural biomaterials include collagen, fibrin, alginate, and chitosan that can be sourced from tissues and cells [28–30]. Alternatively, **decellularized ECM (dECM)** offers the advantage of

manipulation of small volumes of fluids in channels with dimensions from ten to hundreds of micrometers.

Organoids: 3D culture models that are distinct from spheroids because they originate through the proliferation and self-organization of a progenitor cell source that generates a cluster of cells resembling the architecture and functionality of the native tissue they derive from.

Patient-derived tumor xenografts: preclinical cancer models where tissue or cells from a primary tumor of a patient are implanted into an immunocompromised mouse.

Photopatterning: microfabrication technique based on photo-crosslinkable bioinks that polymerize rapidly in the presence of light, which is used to produce porous scaffolds and other 3D constructs.

Spheroids: multicellular tumor spheroids are dense and homogeneous 3D constructs that comprise cancer cells alone or combined with other cell types.

Tumor microenvironment (TME): dynamic environment composed of stromal cells (fibroblasts, endothelial cells, immune cells, mesenchymal stem cells), other nonmalignant host-tissue cells, blood and lymphatic vessels, and cancer cells surrounded by ECM.

Tumor stroma: nonmalignant cells present in the TME of solid tumors, including connective tissue, blood vessels, and inflammatory cells.

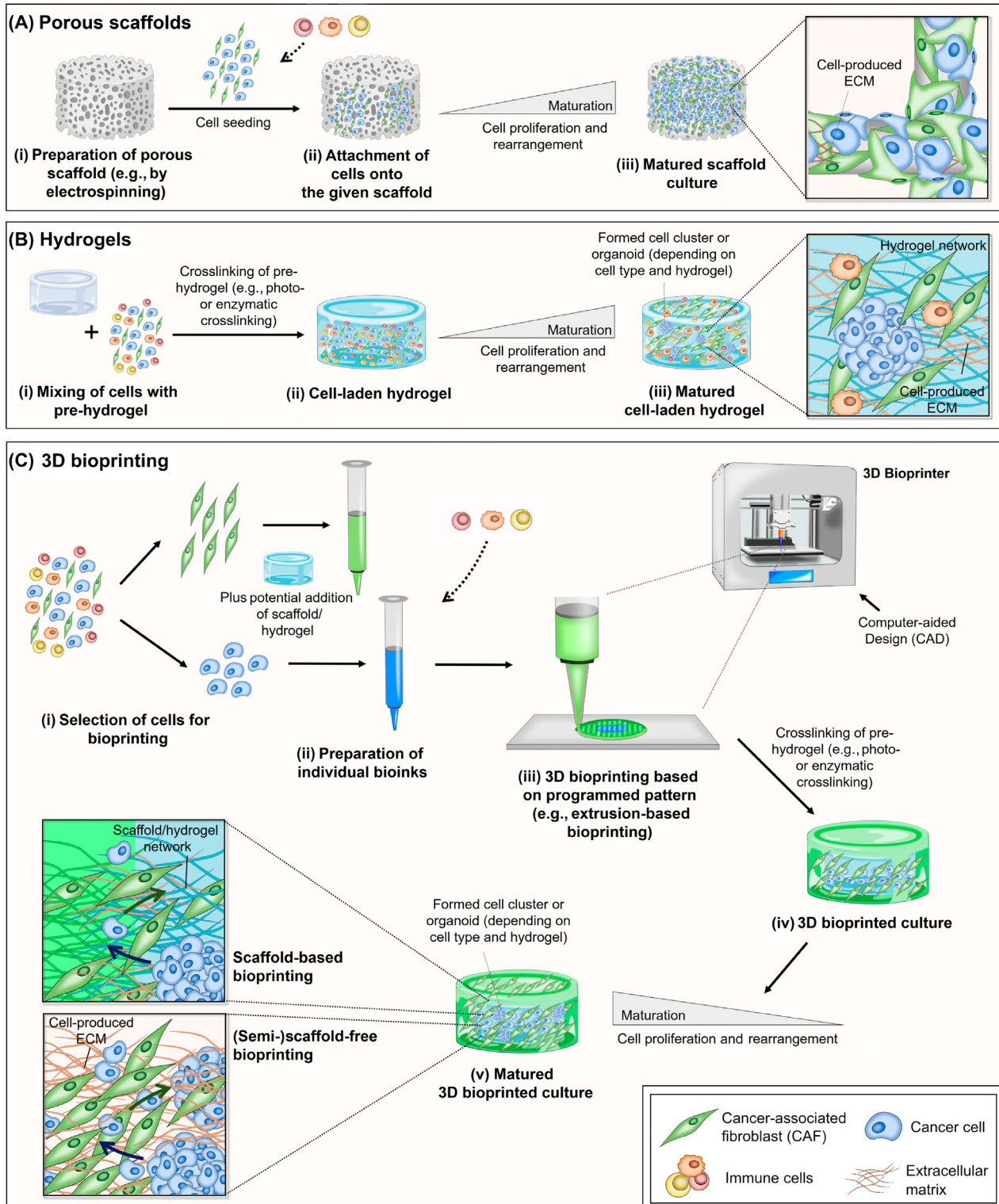
Table 1. 2D versus 3D Cell Culture Methods

Characteristics	2D	3D	Refs
Cell morphology	Altered cell shape, usually flat and elongated; loss of epithelial cell polarity	The natural cell shape and polarization is preserved; cells grow in 3D aggregates	[15]
Gene expression	Cell adhesion-, proliferation-, and survival-related genes are usually modified	Accurate representation of gene expression patterns	[16,85,86]
Cell proliferation and differentiation	Cell differentiation is poor and proliferation occurs at an unnaturally rapid pace	Cells are well differentiated; proliferation is realistic depending on 3D matrix interactions	[15]
Cell interactions	Deprived of cell–cell and cell–ECM interactions, no cell niches are created	Cell junctions are common and allow cell communication	[14]
Tumoral heterogeneity	Basic; all cells receive the same amount of nutrients; inaccurate replication of the TME	Better approximation and representation of the TME; nutrients are not equally supplied	[4,51]
Response to stimuli	Inaccurate representation of mechanical and biochemical cues	Cells grow in a 3D environment and receive stimuli from all directions that properly represent <i>in vivo</i> stimuli	[16,85]
Reproducibility	Highly replicable	Difficult to replicate some conditions	[4]
Analysis and quantification	Easy interpretation of results; better long-term cultures	Difficult to analyze data, especially with multiple cell types or when in spheroid/organoid conformation	[4,17]
Cost	Cheaper for large-scale studies	More complex and expensive techniques	[18,51]

recreating natural biochemical environments without compromising the tissue-specific architecture and the ECM, thereby generating scaffolds that have biochemical and structural cues similar to those present *in vivo* [31,32]. Most commonly used ECM substitutes, such as **Matrigel**, incorporate undefined and highly variable factors that can affect the experimental results and the reproducibility of the model [33]. Given the close resemblance to the native matrix structure, cell–ECM interactions can be more easily replicated in dECM-based models after cellularization. In addition, dECMs are promising alternatives to better control the TME *in vitro*, and have advantages over scaffolds that focus only on individual ECM components and not on the ECM environment as a whole [34]. The decellularization process, however, has its limitations because it is challenging to ensure tissue intactness after treatment with detergents and enzymes.

With these considerations in mind, multiple modifications of scaffold-based cell culture supports have been optimized for tumor modeling. A tissue matrix scaffold (TMS) using native ECM has been developed to overcome cell culture methods that do not mimic the biophysical/biochemical properties of the ECM [35]. This *in vitro* model consists of a multilayered tissue culture platform prepared from decellularized mouse mammary tissue. Cancer and stromal cells are cultured in a compartmental fashion that induces the expression of intracellular and extracellular biomarkers of breast cancer cells, thus confirming correct tumor growth and proliferation. This TMS therefore mimics the structure of the mammary tissue while providing a simple-to-use tool for screening specific tumor biomarkers [35].

In a different approach, a 3D tumor model was developed by generating anisotropic collagen scaffolds seeded with adipocytes and tumor cells [30]. This model allowed examination of adipocytes in the tumor stroma by culturing breast cancer cells in collagen pores aligned perpendicular to the surface, thus reflecting the *in vivo* microenvironment in which the ECM organizes in an anisotropic 3D spatial configuration. The invasion of tumor cells into the stroma was compared between two different cell lines overexpressing either Wnt1 or Her2. The presence of adipocytes increased the migration of both cancer cell types, and promoted cancer cell invasion, while reducing the overall number of migratory cells, thus demonstrating the heterogeneity of cellular behavior in this model [30].



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Scaffolds can provide a proper ECM-mimicking environment for culturing cells and have advantages over 2D models and *in vivo* models. Scaffolds offer an inexpensive and easily analyzable platform which has tunable and instructive properties that can recapitulate relevant biochemical and structural cues [26]. One study showed a significant difference in the levels of gene expression affecting ECM remodeling, namely processes associated with cell adhesion and tumor growth, and this resulted in increased radio- and chemoresistance in the 3D Matrigel model compared with the 2D culture, which highlights the poor reproduction of *in vivo* findings in 2D models [36]. Furthermore, scaffolds can be applied in most current 3D *in vitro* models, and have been used to produce complex **3D bioprinted** models, to induce the assembly of cell **spheroids**, and to promote the 3D culture of cells in microfluidic platforms, as explained in detail in the next sections.

3D Bioprinting

The use of scaffolds in the fabrication of 3D *in vitro* models has paved the way for novel techniques such as 3D bioprinting that use such scaffolds to create more complex models with well-defined architecture, composition, and high reproducibility (Figure 2C) [37]. Cell printing is an emerging approach for 3D cancer cell patterning that facilitates the control of spatial and temporal distribution of cells [3]. Common 3D bioprinting techniques include extrusion-, inkjet-, and stereolithography-based bioprinting, as well as laser-assisted and electrospinning-based bioprinting (Box 1) [38]. The process of printing must avoid damaging pressure/heat sensitive fluids, especially when printing living cells [3]. Therefore, the choice of biomaterial must consider its biocompatibility, the shape-fidelity of the material, and the level of instructiveness required.

3D bioprinting has recently been used to form multicellular structures consisting first of a breast cancer cell core surrounded by human mammary fibroblasts and umbilical vein endothelial cells (HUVECs). Then, primary human subcutaneous preadipocytes and MSCs were incorporated, which led to the formation of a more reactive desmoplastic TME. The same model was then tested for pancreatic ductal adenocarcinoma (PDAC), showing that transforming growth factor (TGF)- β -mediated activation of pancreatic stellate cells (PSCs), precursors of CAFs in pancreatic cancer, enhances tumor cell migration [37]. This bioengineered 3D model enabled the incorporation of cancer cells into a complex microenvironment where interactions between tumor and stromal cells, ECM deposition, and self-organization of the tissue could be observed.

3D printed models also allow 4D manipulation of variables – with time as a fourth dimension – which is crucial for evaluating the dynamics or kinetics of GFs, drugs, or the metastatic spread of tumor cells over time. As a representative example, bioprinted 3D tumor constructs were developed to recapitulate the TME that leads to metastatic dissemination of lung cancer [39]. Bioprinted models were assembled with tumor cells, an endothelialized microchannel, a fibroblast-laden natural hydrogel, and 3D printed programmable release capsules (triggered by

Figure 2. Cell/Extracellular Matrix (ECM)-Based 3D Models. Schematic representations of different cell/ECM-based models describing the key steps and characteristics of each culture. (A) Culture of cells within a porous scaffold starting with (i) the fabrication of a scaffold, followed by (ii) seeding of cells [with potential to seed (attaching) immune cells into the scaffold] and subsequent attachment of cells onto the scaffold material. After maturation (including cell proliferation, rearrangement, and ECM production) the matured scaffold culture (iii) is fully covered by the seeded cells (square panel). (B) Culture of cells within a hydrogel starting with (i) mixing cells with a precursor hydrogel, followed by (ii) crosslinking of the hydrogel (e.g., by enzymatic or photo-crosslinking) to obtain a stable cell-laden hydrogel construct. (iii) After cell proliferation and rearrangement (maturation) the matured hydrogel culture might include cell clusters of rearranged cells as well as cell-produced ECM while the original hydrogel network remains (square panel). (C) 3D bioprinting-based cell culture starting with (i) the selection/sorting of cells for bioprinting, and (ii) preparation of a cell-laden bioink (often mixture of a precursor hydrogel and cells). Next, (iii) the bioink is deposited in a preprogrammed pattern (computer-aided design, CAD) using a 3D bioprinter, followed by (iv) crosslinking of the hydrogel to obtain a stable culture. (v) Depending on the nature of the bioink and the aim of the study, different bioprinted cultures can be obtained after maturation, including a scaffold-based culture (hydrogel remains as part of the culture), scaffold-free culture (no hydrogel was initially used in the bioink, 3D culture is mainly based on strong ECM production by cells), or a semi-scaffold-free culture [hydrogel is removed (e.g., enzymatic degradation) after the cells produce sufficient ECM during maturation to maintain a 3D structure (square panels, arrows indicate migration among printed patterns)].

Box 1. Microfabrication Techniques to Produce 3D Constructs and Microfluidic Devices

Over the past decades, multiple 3D bioprinting methods have been improved to allow the design of complex cell-laden 3D constructs. Each of these technologies is application-specific and the choice depends on the type of bioink used [40,87]. Extrusion-based bioprinting demands viscous bioinks that are dispersed by a syringe, which releases the bioink onto a collector plate, followed by chemical, thermal, or photo-crosslinking [87]. Inkjet-based bioprinting is based on inkjet printers that can layer individual droplets of bioink, thus generating patterned layers [87,88]. Stereolithography-based bioprinting involves digital micromirror arrays that are used to selectively solidify layer by layer light-sensitive bioinks by photopolymerization, thus allowing faster bioprinting speeds [38,87,89]. In laser-assisted bioprinting, a high-intensity laser is used to deposit less-viscous bioinks for cell patterning, thereby preserving high cell viability. Despite its cell-compatibility, this is the most complex and expensive method of printing [40,90]. Finally, electrospinning-based bioprinting combines extrusion-based bioprinting and a high-voltage power supply to produce 3D fiber constructs [38,91]. If the goal is to emulate the TME, the biomaterials selected should mimic the ECM and display viscoelastic properties that enable the formation of stable 3D constructs. Commonly chosen materials include Matrigel, other natural biomaterials (e.g., gelatin, fibrin, collagen, alginate, chitosan, hyaluronic acid), and synthetic polymers (e.g., PEG, PCL) [40,87].

Microfluidic chips are often fabricated by soft lithography – the desired patterning is printed on silicon wafers by photolithography, followed by replica molding of the silicon wafer with a liquid polymer, usually PDMS [64]. PDMS is commonly used in microfluidic devices because it is easy to handle, inexpensive, gas-permeable, and transparent, thus allowing real-time, high-resolution optical imaging [70]. These chips can also be fabricated by, for example, micromolding, microetching, laser etching, injection molding, photopolymerization, and 3D printing [64]. The choice of the fabrication method depends on the application desired and the type of biomaterials used. Most microfluidic platforms designed for cancer research are additionally modified with matrix coatings and/or involve scaffold-based materials to better mimic the native microenvironment of the cells. Tumor cells are typically seeded in a matrix, such as Matrigel, or in the form of tumor cell spheroids/organoids.

laser irradiation) as the sources of chemical gradients. Guided cell migration and angiogenesis were assessed, and the results showed that epidermal growth factor (EGF) released from the capsules actively promotes the proliferation of lung cancer cells and guides their migration [39]. This model enabled precise placement of cells and spatiotemporal control of molecular gradients that locally modulate dynamic cellular events. In general, the fabrication of spatially defined 3D *in vitro* models has been improved by 3D bioprinting techniques. The microscale resolution, high precision in forming 3D constructs, the ability to use multiple materials, and commercial availability have potentiated its utilization. Common limitations, however, include slow printing speeds, development of nontoxic and printable bioinks, and insufficient reproducibility to create standard models [38,40].

Cell-Based 3D Models

Solid tumors are usually surrounded by stromal cells that interact with each other and with cancer cells (Figure 1), thus influencing tumorigenesis and even resistance to anticancer therapy. As one of the most prominent cell types within the tumor stroma, CAFs play a crucial role in carcinogenesis. CAFs are usually identified as α -smooth muscle actin (α -SMA)-positive cells. In contrast to non-cancerous myofibroblasts, CAFs do not revert back to their inactivated state and overexpress platelet-derived growth factor (PDGF) receptor- β , which in turn supports their own proliferation [41]. CAFs interact actively with cancer cells, and not only enhance their survival and invasion capacity but also remodel the ECM and stimulate the proliferation/growth of tumor cells by metabolic cooperation [42,43]. For example, CAFs take up and metabolize extracellular lactate and then export pyruvate that cancer cells use for their metabolism [12]. In addition, CAFs increase the collagen density in tumor tissues, resulting in high matrix stiffness, cell proliferation, as well as hypoxia – through the procarcinogenic activity of TGF- β [41].

MSCs communicate with cancer cells and influence both their behavior and drug resistance. Many studies have confirmed the interaction between MSCs and breast cancer cells [44,45]. MSCs have homing ability to the breast cancer tissue and can release GFs that stimulate growth and **epithelial–mesenchymal transition (EMT)** of cancer cells. MSCs also alter the immunocompetence of the

TME and induce drug resistance of cancer tissues, and have recently emerged as potential targets for anticancer therapy [46].

The tumor stroma is enriched with various immune cells such as tumor-associated macrophages (TAMs), lymphocytes, natural killer (NK) cells, and DCs that are crucial for tumor control. Regulatory T cells, MDSCs, and M2 type TAMs contribute to immunosuppression. By contrast, CD8⁺ T lymphocytes are responsible for destroying cancer cells by detecting their surface antigens. However, as a result of interactions with TAMs, CAFs, and cancer cells, these T cells become suppressed, exhausted, or are unable to reach the tumor parenchyma, thus allowing cancer cells to proliferate [47]. Within the TME, monocytes are recruited and differentiated into TAMs via a process governed by cancer cells [48]. In turn, TAMs promote angiogenesis [by releasing vascular endothelial growth factor (VEGF) and interleukin (IL)-8] and ECM remodeling (via MMP production) [49]. Furthermore, TAMs secrete anti-inflammatory cytokines (IL-10, CCL22, TGF- β) which suppress adaptive immunity and are therefore attractive targets for immunotherapy [7].

Angiogenesis – the formation of new blood vessels – is a naturally occurring phenomenon that supports the nutrient and oxygen requirements of growing solid tumors, promoted by the release of VEGF from cancer and stromal cells. Vascular endothelial cells and pericytes are the main cell types that line blood vessels, and generate abnormal, tortuous in shape, highly fenestrated, and leaky blood vessels. These characteristics induce cancer cell invasion, an important step for the establishment of metastases. Owing to its essential role in the TME, the relationship between endothelial and tumor cells has been a relevant target of multiple anticancer therapies [9].

Cancer-associated adipocytes (CAAs) interact with tumor cells by releasing various GFs that can enhance cancer progression. In breast cancer, CAAs secrete chemokines CCL2 and CCL5, inflammatory factors such as IL-1 β and IL-6, VEGF, tumor necrosis factor (TNF- α), and adipokines such as leptin [50]. All these factors can modify the behavior and stimulate the tumorigenic potential of breast cancer cells.

In contrast to scaffold-based culture methods, cell-based 3D models consist of cell aggregates that usually produce their own intrinsic ECM. These models have emerged with the prospect of closely representing the physiological composition of tumor organization *in vitro*, as well as the tumor–stroma interactions that play a crucial role in tumorigenesis.

Spheroids

Spheroids are one of the most used 3D models for the study of tumor biology, especially for testing anticancer drugs. Spheroids accurately recapitulate important tumor features including cellular heterogeneity, cell signaling pathways, cell–cell/cell–ECM interactions, gene expression patterns similar to those of *in vivo* conditions, and a tumor morphology composed of different cell layers [51]. Various techniques can be used to produce cell spheroids (Box 2). Most methods are relatively simple and can achieve high-throughput capabilities with flexibility to integrate multiple cell types or different types of gradients (Figure 3A).

Owing to the limited diffusion of nutrients and oxygen, larger spheroids (500 μ m in diameter) mimic the microenvironment of micro-metastases and avascular tumors, and thus represent a proper model for studying the effects of hypoxia on cancer development [52]. This physiochemical gradient generally induces the formation of three concentric zones on a spheroid – an anoxic core in the center containing necrotic cells, a middle hypoxic zone that has a low concentration of oxygen and nutrients, and an outer zone containing highly proliferative cells [53].

Box 2. Methods for Generating Spheroids and Tumor Organoids

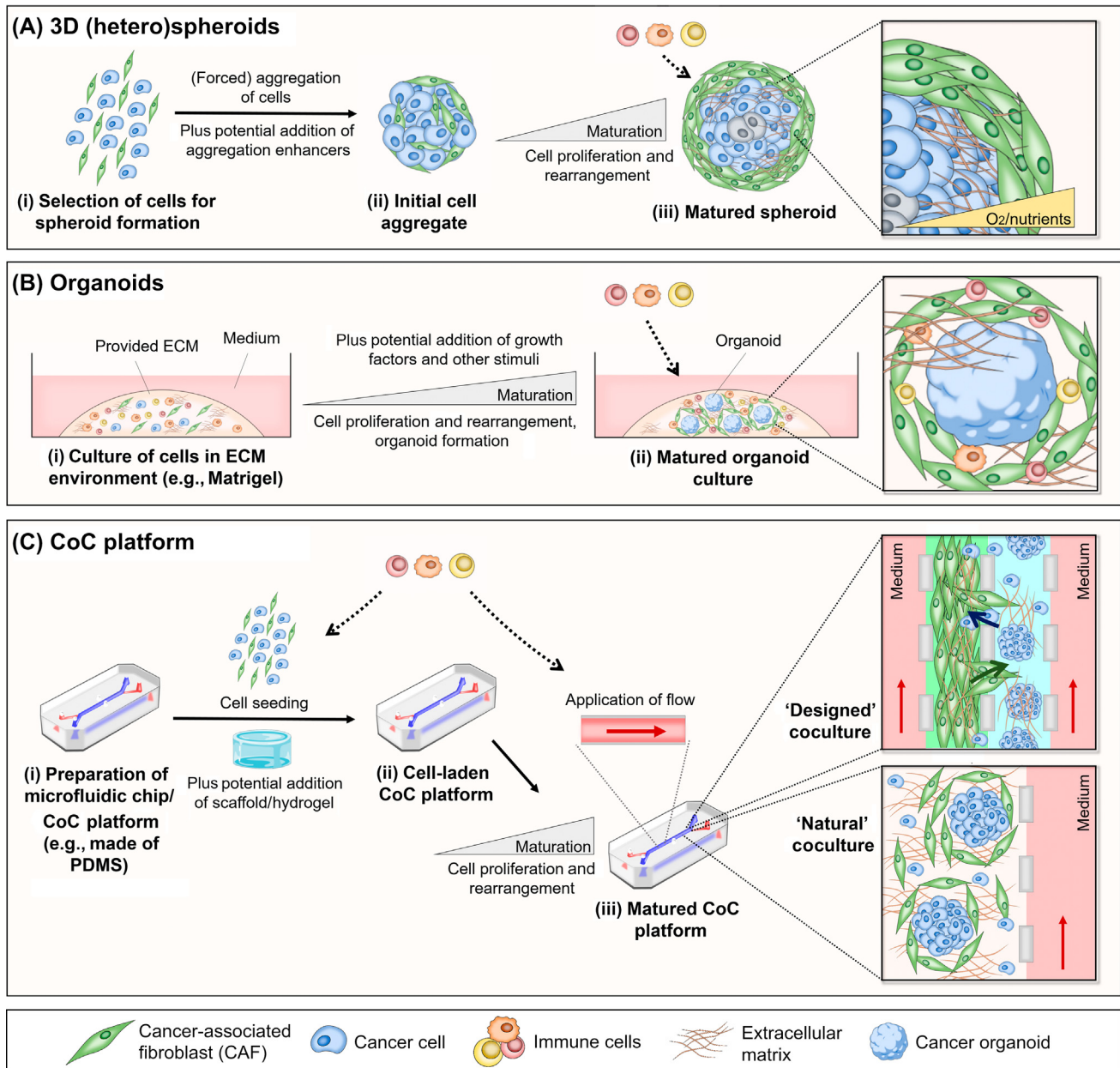
Techniques to produce spheroids include hanging drop methods, spontaneous formation on low-attachment surfaces, magnetic levitation, 3D bioprinting, micropatterned plates, spinner flasks, and matrix encapsulation [53]. The choice of technique depends on the application and the inherent advantages and limitations of each method. Microfabricated structures usually provide spheroids that are homogenous in both size and number, whereas 3D bioprinting and magnetic assembly allow better precision and the incorporation of multiple cell types. Spinner flasks and hanging-drop methods are normally simpler to use and easy to set-up, but have limitations in terms of size homogeneity [57]. Cell sources can be from patients, xenografts, or cell lines [5]. An important aspect to consider is uniformity in size and homogeneity to ensure that the results are not influenced by these factors [53]. Recent progress has directed the development of high-throughput methods for uniform spheroid assembly and continuous perfusable culture that secure reproducibility [92]. For example, a microfluidics approach was established to produce multicellular alginate spheroids that have tumor cells in the core and stromal fibroblasts in the shell for high-throughput drug testing [93].

Tumor organoids can be generated from patient/mouse-derived tumor cells or by engineering induced pluripotent stem cells (iPSCs) followed by gene editing to transform a normal organoid into a cancer organoid [33,59]. In general, growing tumor organoids directly from cancer tissue seems to be more practical than to use iPSCs because the efficiency of producing patient-derived iPSC organoids depends on the type of cancer. In addition, iPSCs are associated with loss of genetic heterogeneity and outgrowth of tumor subclones, and this can be related to the presence/absence of particular oncogenic mutations [33]. Organoids are grown on a matrix, normally Matrigel or collagen [5,33]. Human tumor organoids have been successfully developed from colon, pancreas, prostate, breast, gastric, cerebral, lung, esophageal, bladder, ovarian, kidney, and liver tumor tissue [60,94].

Although more time-consuming and expensive than 2D cell culture, spheroids are a widely used and cost-effective 3D culture method than can be associated with 3D bioprinting technologies, microfluidics, and scaffold-based platforms to generate more physiologically representative tumor models.

Tumor cell spheroids are especially useful for studying cancers that form tumor embolus, or a closely packed tumor cell cluster, as happens in inflammatory breast cancer [53]. Using a 3D human breast cancer spheroid model, angiogenesis sprout formation was found to be dependent on VEGF and fibroblast growth factor (FGF) [54]. Furthermore, fibroblasts mediated endothelial sprout formation through the expression of MT1-MMP, which is essential for the migration of endothelial cells. Moreover, spheroids incorporate various levels of TME complexity. Aiming to analyze the influence of ECM stiffness on breast cancer, stromal cell-laden microwell arrays were formed with tunable stiffness to generate spheroids [55]. The preadipocytes were mixed with hydrogel precursor solutions and micromolded into the desired microwell pattern. Then, breast cancer cells were seeded, and formed tumor spheroids in the microwells. The cancer cells interacted with stromal cells, and inhibited preadipocyte differentiation and maturation only in high-stiffness tissue. These findings could not be replicated in a 2D monolayer culture system, highlighting the importance of a 3D environment to mimic TME interactions [55].

Although macrophages play a crucial role within the TME, their natural ECM remodeling behavior is particularly challenging to integrate into spheroid-based models. Nonetheless, different strategies have been applied in the recent years to better understand the role of macrophages in the TME using spheroids. For instance, a 3D culture model was developed based on alginate micro-encapsulation to form tumor spheroids of non-small cell lung carcinoma (NSCLC) cells with CAFs and monocytes, cultured in spinner-flasks [29]. The spheroids secreted cytokines (e.g., IL-5, IL-10, IL-13, CCL22, CCL24, and CXCL1), collagen, fibronectin, and MMP1/9, which created an immunosuppressive TME. The interactions of the included monocytes with CAFs and cancer cells led to their polarization into M2-like macrophages, thus resembling the TAM phenotype in NSCLC [29]. Whereas this model includes monocytes in the initial phase of spheroid formation by encapsulating them into the alginate capsules, a different strategy can be the infiltration of monocytes once cancer cell/fibroblast spheroids have already been formed. For instance, following this strategy, pancreatic



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Figure 3. Cell-Based 3D Models and Microfluidic Cancer-on-a-Chip (CoC) Models. Schematic representations of different cell based as well as microfluidic models describing the key steps and characteristics of each culture. (A) Fabrication of 3D (hetero)spheroids starting with (i) selection of cells (usually cells that show high cell–cell contact or high deposition of extracellular matrix, ECM) followed by (ii) (forced) aggregation of cells using, for example, centrifugation into microwells, or hanging-drop or low-adhesion cultures to obtain an initial cell aggregate. To aid aggregation different enhancers (e.g., Matrigel) can be added to provide initial ECM. After cell proliferation, rearrangement, and ECM deposition (maturation), (iii) dense spheroids are formed that usually present an oxygen or nutrient gradient towards the center of the spheroids (square panel). Immune cells can be added to the matured spheroids to allow penetration of immune cells into the spheroid culture. (B) Fabrication of organoids starting with (i) culture of cells in a hydrogel (ECM) environment [e.g., using Matrigel or basement membrane extract (BME) type 2]. After maturation, (ii) organoids are formed that are surrounded by other cells added to the culture which proliferate, rearrange, and produce cell-natural ECM (square panel). Immune cell penetration can be mimicked by adding the respective cells to the initial hydrogel or to the culture medium. (C) Culture of cells in a microfluidic CoC platform starting with (i and ii) seeding of cells into the prepared CoC platform. Potential combination of cells with a hydrogel to achieve a 3D culture as well as seeding of (attaching) immune cells

(Figure legend continued at the bottom of the next page.)

cancer spheroids consisting of different cancer cell lines (PaTu-8902, BxPc3, HPAC, and MiaCaPa-2) and MRC-5 fibroblasts were developed using low-adhesion plates [56]. After successful formation of the cancer cell/CAF spheroids, the spheroids were incubated with monocyte-containing medium. These monocytes, isolated from blood samples, were able to penetrate into the spheroids, similarly to monocyte infiltration into *in vivo* tumors, and eventually became polarized towards an M2 phenotype. Both strategies confirmed that the interplay between the ECM, tumor cells, and stromal and immune cells promotes the activation of TAMs, thus mimicking aggressive tumor stages.

Although spheroids have advanced our understanding of tumor biology, there are some limitations related to reproducibility because of poor uniformity in size/morphology, which hinders the development of standard models [57]. In addition, some techniques are associated with low throughput and with difficulty in retrieving cells for analysis [57].

Organoids

Organoids are generated by the proliferation and self-organization of a progenitor cell source (Box 2), and can closely mimic the 3D structure and architecture of the tissue from which they were derived (Figure 3B) [33]. Because tumor organoids usually develop from a single cell into a 3D construct, and follow the different development stages of natural tumors, such organoids are capable of retaining the natural cancer cell heterogeneity of the native tumor to a greater extent, thus preserving the pathophysiology of the tumor *in vitro*, including the genetic and phenotypic features of that specific tumor [58]. Whereas spheroids are formed by the (forced) aggregation of multiple cells into a 3D construct, tumor organoids develop a 3D shape on their own based on their genetic programming, and this more closely represents the actual development of a tumor, thus giving them an advantage over spheroids or other 3D cultures. Moreover, organoids can be efficiently cultured with a patient's own cells, and are thus a better alternative to expensive and labor-intensive **patient-derived tumor xenografts** [59], and are also more cost-effective and physiologically relevant than animal models. However, working with organoids has some drawbacks: the process is more time-consuming, there is difficulty in reaching *in vivo*-like maturity in some models, the variability can be high between experiments, and there is a lack of vasculature and stroma [60].

Given their ability to retain crucial tumor features, organoids have become a suitable platform for studying tumor–stroma interactions. With respect to CAFs and pancreatic cancer, it has been established that coculture of murine PSCs and PDAC tumor organoids leads to CAF differentiation into two subtypes – one with elevated expression of α -SMA (myofibroblast-like CAFs) and the other with high expression of IL-6 (inflammatory CAFs) [61]. This differentiation leads to the production of desmoplastic stroma, and provides evidence of CAF heterogeneity in PDAC and cooperative interactions in the cocultures.

In spheroid-based models, multiple cell types can be cultured together to generate multicellular heterotypic spheroids. By contrast, organoid models usually only contain progenitor cells of epithelial origin, hence there is a lack of an immune-competent microenvironment and stromal components in tumor organoid models [59]. This problem can be overcome by coculture with stromal cells. In one study, pancreatic cancer organoids were established from patient-derived

is possible. (iii) During or after maturation a flow can be applied in the chip to assist cell proliferation and rearrangement as well as to allow the addition of immune cells or therapeutics. Depending on the chip design, two different culture models can be obtained, including a 'designed' coculture (cells are cultured separately but in close proximity to allow migration: the green and blue arrows indicate migration, the red arrow indicates flow) or a 'natural' coculture (cells are seeded together and rearrange: the red arrow indicates flow) (square panels). Abbreviation: PDMS, polydimethylsiloxane.

tumor cells and were then cocultured with CAFs and human T cells. Activation of CAFs and tumor-dependent infiltration of lymphocytes was observed [62]. It was also demonstrated that organoids derived from cell lines cultured in a monolayer or passaged as mouse xenografts are phenotypically distinct from primary organoids that retain the tumor architecture, cell–cell interactions, stemness, and cellular heterogeneity. This demonstrates the advantages of directly culturing cells in 3D models over 2D cultures where cells rapidly lose the native tissue phenotype.

3D bioprinting can also be used with organoids to form sophisticated organoid culture systems [59]. For instance, a novel breast cancer organoid model was developed that was composed of breast cancer cells and a self-gelling hydrogel derived from ECM of decellularized rat or human mammary tissue [63]. Structural and signaling profiles were retained within these dECM hydrogels, and these elicited distinct responses when cultured with normal mammary or breast cancer cells, highlighting the biologically relevant features of this multicellular culture model. In addition, the disposition of cells without any predetermined organization typically may lead to uncontrollable cellular behaviors [63]. Thus, the combination of 3D bioprinting with organoids has the potential to promote the appropriate spatial arrangement of cells in complex 3D constructs while maintaining the hierarchical-like architecture of TME and increasing the reproducibility of the model.

Microfluidic Models

Microfluidics has revolutionized our ability to mimic the natural biophysical/chemical conditions of cells in *in vitro* models. The goal of microfluidics is to build platforms that can model the (patho) physiological functions of tissues and organs – so-called organ-on-chip devices (Figure 3C). These platforms consist of a network of microfluidic channels that allow the culture of cells that are continuously perfused. The main advantage of microfluidics is the ability to design complex 3D culture systems in which various parameters can be modified and controlled independently. The mechanical forces applied, the orientation of tissue interfaces, the types and localization of cells, and the chemical gradients can all be specifically controlled, thus allowing optimized levels of cell survival [64]. Moreover, these devices use microscale volumes, making them less expensive to use compared with other 3D culture methods and bioreactors [1], while also allowing high content and high-throughput screening with increased controllability [4,17,51]. Some limitations include the specialized skills that are necessary to fabricate chips (Box 1) [64]. In this context, the fast development of new fabrication techniques, such as 3D bioprinting, has led to a general decline in the cost and the specialized work involved in fabricating these chips [65–67]. In addition, these models should have better reliability and robustness because they should not be affected by external technical impairments such as air bubbles. Edge effects and high shear stress can also affect the performance and consistency of the device by hindering laminar flow in channels, which can harm cells and/or affect their distribution, resulting in inconsistent results which may compromise the predictive value of the platform [68]. Finally, there is a need for new materials to fabricate the chips because polydimethylsiloxane (PDMS), the most commonly used material, can nonspecifically absorb small molecules [69,70].

Multiple cell types can be cultured in a microfluidic chip to analyze specific interactions, which is particularly interesting when investigating communication between cancer and stromal cells. As an elegant example, a perfusable microfluidic platform was developed to assess breast cancer–immune cell interactions [71]. Breast cancer cells, monocytes, and HUVECs were spatially confined within a gelatin hydrogel by 3D **photopatterning**, and T cells were then dispersed within the medium. The results showed greater T cell infiltration when monocytes were present in the culture, and also when higher levels of hypoxia were emulated by using tumor spheroids instead of dispersed cancer cells [71]. These findings demonstrate the important role of microfluidics in

generating heterotypic 3D models where different cell types can be cultured in a dynamic microenvironment and specific tumor–stroma interactions can be studied.

Given the possibility of controlling multiple gradients, microfluidic devices are often employed to analyze the effects of GFs or drugs in a biomimetic microenvironment. To mimic the human colorectal TME and reconstitute functions of the microvascular tissue, a 3D microfluidic chip-based *in vitro* model was fabricated [72]. The platform was inspired by radial drug penetration (diffusion of small molecules from the outer boundaries into the central core of the tumor) into solid tumors, and it evaluated in real-time the interactions between colorectal cancer cells and endothelial cells that infiltrated the VEGF-infused tumor core [72].

Stroma-driven ECM remodeling is a crucial consequence of tumor–stroma activation that sustains cancer progression. As a way to replicate ECM activation *in vitro*, a tumor-on-a-chip model with a stromal compartment consisting of fibroblast-assembled ECM and breast cancer cells was designed [73]. The results revealed that cancer cell invasion led to the activation of CAFs and overexpression of fibronectin and hyaluronic acid in the ECM. Real-time analysis of collagen remodeling showed that normal fibroblasts produced a finer structure of collagen bundles than did CAFs, supporting earlier findings of human biopsy studies. Therefore, owing to its optical accessibility, this platform provided a unique way to monitor the switch between healthy and pathological stroma *in vitro*, and represents an alternative to the ectopic *in vivo* experiments that are typically used to analyze such events. The design of the microfluidic chip also allowed precise control of cell confinement and interaction, continuous perfusion, and assembly of cell-produced ECM.

Innovative models have recently been developed that combine tumor organoids/spheroids and microfluidic chip systems. By incorporating organoids into a microfluidic device, organoids-on-a-chip inherit the combined benefits of both microfluidics and 3D organoid models, thus providing a unique way to study tumor–stroma interactions and their systemic effects [69,74–76]. For instance, a NSCLC organ-on-a-chip model was developed that recapitulated organ microenvironment-specific cancer growth/dormancy [77]. It was demonstrated that physical cues related to breathing motions were associated with alterations to tumor cell growth, invasion, and drug resistance, and these appear to be mediated by changes in signaling through epidermal growth factor receptor (EGFR) and MET protein kinase [77]. In another study, a 3D microvascular network of endothelial cells was generated in bone- or muscle-mimicking microenvironments with osteo-differentiated cells or smooth muscle MSCs, respectively [78]. Endothelial cells displayed morphological features similar to *in vivo* conditions, contrasting to poorly aligned cells grown in 2D cultures or deprived of flow. This microfluidic device not only promoted the perfusable culture of four different cell types but also proved to be an appropriate platform for monitoring cancer cell extravasation in real time. The extravasation rates were substantially higher in the bone-mimicking microenvironment, whereas microvasculature permeabilities were higher in myoblast-containing matrices, and this demonstrates how distinct microenvironments can influence cancer progression.

Improving Our Understanding of Multifactorial Interactions

Advances in 3D culture models allow better recapitulation of the complex TME *in vitro*. However, these models have several limitations that need to be assessed before using them to derive conclusions. One of the most crucial aspects is the introduction of patient heterogeneity. Clinical tumors have high heterogeneity in terms of various mutations in malignant cells, the tumor stroma content, and the types of immune cells in the TME. Inclusion of all these variables in *in vitro* models is paramount but also relatively complex because of the continuous availability of patient material,

the long preservation times of cells or tissues, and concerns regarding the reproducibility of cell isolation and culture methods. It is evident that cells isolated from patient samples can rapidly lose their phenotypes in culture, and thus the cells may not properly reflect their former function. For example, CAFs are 'trained' to attain particular phenotypes by their surrounding micro-environment *in vivo* [79,80]. CAFs that are in close proximity to cancer cells present a myofibroblast-like phenotype (myCAF) defined by a high expression of α -SMA, whereas CAFs distant from cancer cells barely express α -SMA but secrete high amounts of inflammatory cytokine IL-6 (iCAF), which contradicts the previous definition of CAFs as α -SMA cells [61]. More recently, a new population of CAFs could be identified that is directly involved in immune processes where they function as antigen-presenting CAFs that have high expression of MHC class II [79]. Given this increased understanding regarding different CAF populations, the controlled environment in 3D *in vitro* cultures might further aid in investigating these different subpopulations or in identifying so far unknown populations. However, novel culture protocols or methods need to be developed that allow the maintenance of the different subpopulations in an *in vitro* environment. The design of such models (e.g., mimicking the exact tissue architecture or the combination of different cell types) can have a significant influence on maintaining different populations such as the proximity of CAFs to tumor cells. The integration of multiple cell types in a single culture model remains challenging because multiple variables (e.g., culture medium composition, culture time duration, the difficulty of retrieving and analyzing cells) need to be adjusted such that all cultured cell types can properly thrive. In particular, most reported models are either deprived of immune cells or are supplemented with limited immune cells such as macrophages or T cells. Nonetheless, the immune landscape of the TME is large, and these cells can influence the function of other stromal cells [47]. Although different studies have attempted to incorporate immune cells into a 3D culture, for instance by letting immune cells penetrate into already formed spheroids [56], the inclusion of different immune cell types in 3D models as well as their continuous renewal has remained a huge challenge in the field.

Recent advances in 3D *in vitro* culture models have enhanced the development of new systems that incorporate the heterogeneous microenvironment to which cancer cells are exposed. 3D bioprinting in combination with scaffold-based models, spheroids, and organoids has supported the creation of highly complex environments for cancer modeling that recreate complex architectures. Regarding organoids, holistic approaches for producing immune-competent tumor organoids are emerging. Instead of adding exogenous stromal cells to epithelial organoids, tumor biopsies are cultured directly together with endogenous stromal cells, and this can hinder long-term culture [81,82]. Furthermore, because cancer is a dynamic disease, organ-on-a-chip devices are being developed to recreate multiorgan interactions – broadening the focus of investigating specific events at a single organ/tissue scale towards systemic-like interactions [69,83]. These platforms could even be personalized with a patient's cells, which could revolutionize the field of personalized medicine [84]. Hurdles to overcome include the validation of these models versus *in vivo* results, improving cell separation/isolation techniques, and ensuring the cell culture fluid/media compatibility when connecting between the multiple organ chips.

Concluding Remarks

Ample evidence over the past decade has highlighted the utmost importance of tumor–stroma interactions in cancer progression, metastasis, and drug resistance. Therefore, understanding the interplay between cancer cells and the surrounding stroma is imperative for developing more effective therapies. The goal is to establish a more mechanistic biological insight about these interactions to possibly reverse the tumor-promoting effects of the microenvironment. For that, biologically relevant 3D *in vitro* models are required. Integration of emerging technologies such as polymeric scaffolds, 3D bioprinting, and organ-on-a-chip platforms has revolutionized

Outstanding Questions

How can patient heterogeneity be introduced effectively into *in vitro* models?

Can *in vitro* tumor models effectively evaluate the efficacy of immunotherapies?

How can the reliability and availability of advanced microfabrication techniques be improved?

How can novel organ-on-a-chip platforms evolve into standard and physiologically relevant models for studying cancer *in vitro*?

How can tumor–stroma interaction models be applied to personalized therapy to patients?

this field. Furthermore, the use of human patient-derived primary cells in these models is highly attractive for the pharmaceutical industry because it would enable examination of compounds that can intervene in tumor–stroma interactions.

In summary, the ability to model cancer *in vitro* has never been greater, thus allowing deeper understanding of the key molecular/cellular pathways related to tumor malignancy. Although some questions remain unanswered (see Outstanding Questions), recent advances in for example, microfabrication technologies have boosted the creation of innovative bioengineered 3D models with great potential to emulate the TME.

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