Recreating the extracellular matrix: novel 3D cell culture platforms in cancer research

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Cancer initiation and progression heavily rely on microenvironmental cues derived from various components of the niche including the extracellular matrix (ECM). ECM is a complex macromolecular network that governs cell functionality. Although the two-dimensional (2D) cell culture systems provide useful information at the molecular level and preclinical testing, they could not accurately represent the *in vivo* matrix microenvironmental architecture. Hence, it is no surprise that researchers in the last decade have focussed their efforts on establishing novel advanced *in vitro* culture models that mimic tumour and tissue-specific niches and interactions. These numerous three-dimensional (3D) culture systems that are now widely available, as well as those still under development, grant researchers with new, improved tools to study cancer progression and to explore innovative therapeutic options. Herein, we report on the emerging methods and cutting-edge technologies in 3D cell culture platforms and discuss their potential use in unveiling tumour microenvironmental cues, drug screening and personalized treatment.

Introduction

Extracellular matrices (ECMs) consist of specific macromolecules and enzymes that provide structural stability and support, as well as functional flexibility to cells, tissues and organs [1]. Matrix integrity guides tissue organization and the fine-tuned vivid matrix turnover during normal conditions, such as development, wound healing and homeostasis [2]. It is well documented that matrix dysregulation and excessive remodelling have been correlated with the pathogenesis and the progression of several human abnormal conditions, including metabolic disorders, fibrosis and cancer, highlighting matrix components as key players in disease development and progression [3].

Recreating the matrix microenvironment in *in vitro* setups to mimic *in vivo* conditions and improve cell behavioural studies has been proven to be a demanding task. The simple, two-dimensional (2D) cell culture systems lack the ability to accurately represent the *in vivo* tissue and tumour architecture, microenvironmental cues and drug response. However, as our understanding on cancer ECM biology and tumour niches advances, so does the available technologies

Abbreviations

2D, two-dimensional; 3D, three-dimensional; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; GAG, glycosaminoglycan; HA, hyaluronan; PA, polylactic acid; PDOs, patient-derived organoids; PDXs, patient-derived xenografts; PEG, polyethylene glycol; PG, proteoglycan; PIC, polyisocyanides.

and methods that allow us to transit from the conventional 2D cell cultures to more advanced three-dimensional (3D) cell culture systems (Fig. 1). These models provide us with new tools that expand the *in vitro* studies and allow us to investigate the initiation and progression of cancer, as well as the development and testing of new therapeutic strategies.

In this article, we report on the most recent advances in 3D cell culture systems, as well as the opportunities they provide to improve cancer research and drug discovery. These approaches may improve preclinical drug testing and facilitate the discovery of novel pharmacological targets.

ECM bioactive effectors

The multitasking ECM is formed by interacting macromolecules and bioactive modulators that upon cell–cell and cell-matrix communication affect cell phenotype and functions [4]. Matrix macromolecules are finely orchestrated to form a 3D dynamic meshwork that can be described as the most important and abundant biomaterial in human organisms. The core ECM network consists of proteins and glycoproteins, such as proteoglycans and glycosaminoglycans (PGs/GAGs), collagens, elastin, laminins, tenascins, cell receptors like integrins and the hyaluronan (HA) receptor CD44, and matrix-degrading enzymes, such as proteases, including matrix metalloproteinases (MMPs) and glycosidases, such as heparanase [2,5,6].

In traditional 2D cultures, cells are typically cultured on flat surfaces that lack the 3D architecture and mechanical properties of native tissues. This can lead to altered cell behaviour and ECM secretion, including changes in ECM composition, organization and mechanical properties, as a result of modified cell signalling pathways and mechanical cues. On the other hand, cells in 3D cultures are typically cultured in structures that better reflect their surrounding environment, leading to more accurate representation of cell behaviour and ECM deposition. As a result, 3D cultures offer a more physiologically relevant model system for studying cell-ECM interactions and tissue development. It is therefore apparent that studies concerning the recreation of the ECM, the expression and synthesis of its biomolecular effectors, its assembly, and organization in 3D cell cultures platforms are a prerequisite for a deeper understanding of the ECM roles in cancer pathogenesis, growth and tumour propagation (i.e. invasion and metastasis). The ultimate goal of the field, however, remains the bridging of the gap between *in vitro* and *in vivo* models.

The functional interactions of matrix bioactive effectors with the conterminous microenvironment are the key regulators in tissue homeostasis and pathological conditions, including cancer [7]. During cancer progression, major components of the provisional matrix reprogram the primary tumour cells and evoke the formation of the premetastatic niche [3]. For instance, HA, the only nonsulfated GAG, is a ubiquitous matrix component comprised of repeated disaccharide units of glucuronic acid and *N*-acetylglucosamine, considered as one of the main players of cancer initiation and progression [8–10]. Owing to this, hybrid 3D
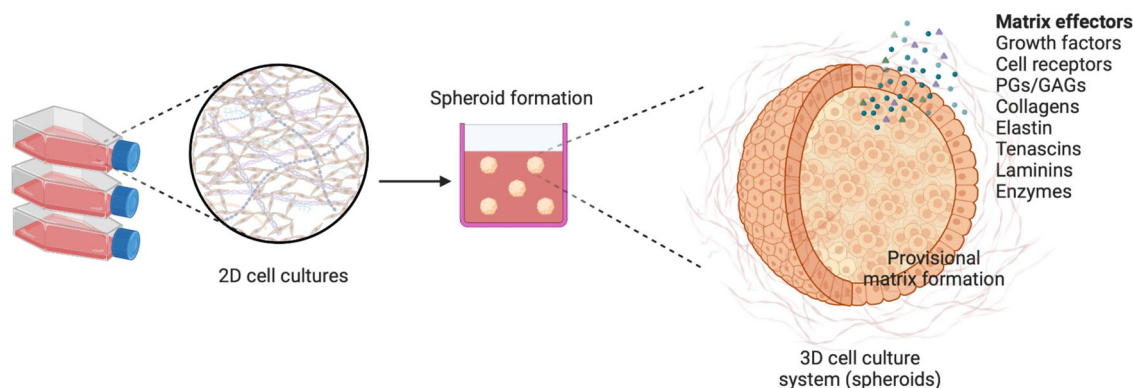

Fig. 1. Transition from conventional 2D cell culture to advanced 3D cell culture platforms. Cells cultured in 2D systems show a flat shape that does not represent the 3D cell morphology, whereas cells cultured in a 3D system, such as in spheroids are integrated into a microenvironment more closely mimicking the *in vivo* conditions and demonstrating a more representative cell behaviour. The cancer cells in 3D spheroids produce a plethora of matrix effectors in the provisional matrix that is formed around the spheroid often in higher bioavailability compared with the secretion of the same matrix effectors in 2D systems. The released matrix effectors in 3D systems including growth factors, cytokines, membrane molecules, etc., are recruited to stimulate cancer cell behaviour in endocrine and/or paracrine manner. Created with Biorender.

biomimetic scaffolds of chemically modified HA have been developed as a liver cancer model [11]. It is reported that the high content of HA in the ECM is essential to create a tissue-like microenvironment favourable to the functions of cancer cells (i.e. proliferation, migration, invasion, etc.). However, the use of native HA has been unreliable due to poor bioavailability, stability and functionality [12]. To this end, biodegradable chemically modified HA-based 3D systems that ensure cell growth, nutrient uptake and the formation of tissue-like structures have been developed. Notably, HA-based 3D culture systems exhibit greater chemosensitivity compared with conventional 2D cultures, as in the case of breast and liver cancer [11,13,14].

Another key component of the ECM, the highly porous collagen (I–XXVIII), forms fibrils, filaments and networks that act as scaffolds for supramolecular assemblies providing mechanical stability and functions. The specificity of collagen to spatial organization and self-assembly has strengthened its use to support the *in vitro* growth of many types of tissues. Depending on the type of tissue, collagen fibrils organize themselves in different ways to form collagen fibres that support specific tissue functions and properties [15]. For instance, type I collagen has been widely used in hydrogel scaffolds. Ovarian cancer cells cultured in type I collagen scaffolds gradually turned to 3D spheroids with increased cell viability and enhanced expression of epithelial-to-mesenchymal transition (EMT) markers [16], while acute lymphoblastic leukaemia Jurkat cells cultured in type I collagen scaffolds showed increased cell proliferation and drug resistance [17]. Moreover, type IV collagen scaffolds increased cell proliferation and invasion of squamous carcinoma cells [18].

Emerging platforms for three-dimensional cultures

For the past several decades, 2D cell culture models have been the gold standard for investigating tumour progression *in vitro*. However, these models display significant limitations in imitating the adjacent niches, both at the structural and molecular levels. Given the influential role of the tumour microenvironment and particularly ECM in cancer initiation and progression, including tissue-specific cell-matrix interactions that remain elusive, the development of new 3D matrix-mimetic tumour models to accurately represent the local microenvironmental cues has become imperative [19]. Ultimately, the central aim is to design, engineer and optimize new culture platforms as to assess distinctive inputs of the tumour microenvironment, all the while having the opportunity to test novel anticancer drugs and predict clinical outcome.

The typical 2D cultures, where cells are grown in monolayers or even the cultures in which the cells are plated on top of a thick layer of different ECM components, are informative and cost-effective but relatively simplistic, as the data generated this way is often non-predictive for *in vivo* applications [20]. Nonetheless, in 3D cultures, cells tend to form complex structures and recapitulate tumour heterogeneity. Moreover, a 3D environment gives researchers the opportunity to better observe morphological and physiological changes, to co-culture different cell populations (i.e. tumour cells, macrophages, fibroblasts, etc.) and emulate interactions and functions similar to the impacted tissue, and to have better overall control of important microenvironmental cues, i.e. temperature, pH and oxygen rate, to name a few [21]. Researchers worldwide are already taking advantage of such 3D culture setups as an interim solution that precedes animal model studies, thus saving both experimental expenses and time, to bridge the gap between *in vitro* and *in vivo* methods.

Most of the widely used 3D culture techniques can be sorted into the following categories, each with their own sets of strengths and limitations: anchorage-dependent (or matrix-based), anchorage-independent (or matrix-free) 3D culture models, as well as hybrid systems, in which already formed spheroids are incorporated into a 3D polymeric scaffold (Fig. 2) [22]. The main 3D cell culture techniques currently in use are summarized and compared in Table 1. Additionally, the development and optimization of a 3D culture platform are based on different main principles; parameters such as the application, the nature of the cells (i.e. selected cell line, primary cells, stem cells, tissue origin), the 3D artificial microenvironment in which they are grown, along with the biomaterials used (i.e. natural, synthetic, etc.), should be considered when choosing the most appropriate technique and culture system [23].

Matrix-based culture systems

In matrix-based models, cells are embedded within a scaffold or matrix, whose physicochemical and mechanical properties will in turn affect their characteristics (Fig. 2). There is a wide range of available bioscaffolds, including hydrogel-based and polymeric-based supports of different origins, natural or synthetic, that offer a dimensionality favourable for the recapitulation of cellular behaviour in the natural microenvironment [24].
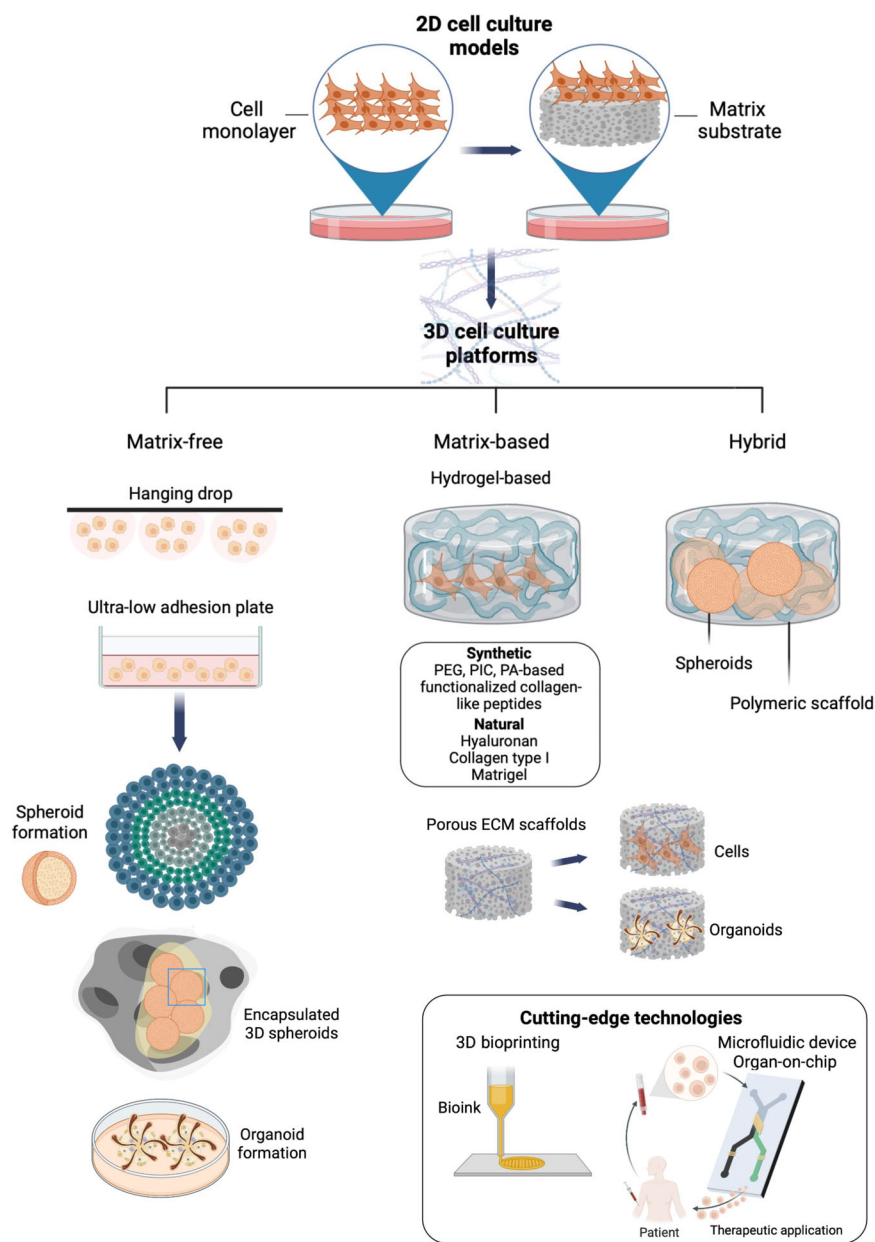
Fig. 2. Schematic representation of major techniques and novel technologies to develop 3D cell culture platforms. Classic 2D methods to study the *in vitro* tumour progression are comprised of cells grown as monolayers or even of cells cultured on top of a thick matrix substrate. The establishment of the role of tumour microenvironment and ECM guided to the development of 3D cell culture systems to mimic the *in vivo* tumour conditions. The main cell culture techniques used to recreate 3D models comprised of matrix-free models (i.e. hanging drop assay, ultra-low adhesion plates, spheroid/organoid formation), matrix-based models (i.e. natural, and synthetic hydrogels) and hybrid systems (i.e. spheroids into 3D polymeric scaffolds). Recent advances on 3D culture platforms led to the development of cutting-edge technologies, such as 3D bioprinting with specific matrix bioinks, as well as microfluidic devices, such as organ-on-chip, that allow high throughput analysis and bring us one step closer to personalized therapeutic approaches. Created with Biorender.

Hydrogels, known as 3D networks of crosslinked hydrophilic polymer chains, can absorb large amounts of water while maintaining their structure [25]. Hydrogels from natural sources, such as collagen I or Matrigel® are the most widely used for 3D cultures. By modulating the physical and mechanical properties of the hydrogels, researchers can alter cell functional properties, activate different signalling pathways and investigate therapeutic responses. For instance, Cavo *et al.* [26] showcased the impact of substrate elasticity

Table 1. Comparison of different 3D cell culture techniques.

Technique	Advantage	Disadvantage
Matrix-based/ Hydrogels	• Widely used in cancer research • Ease of handling • Adjustable physical, chemical, mechanical properties • Possibility for co-cultures • High differentiation potential • Cell–cell and cell–matrix interactions • Drug screening	• Batch-to-batch variability • Prone to biodegradation • Final composition cannot be controlled (Matrigel®) • Possible transmission of pathogens due to animal origin (collagen I, HA)
Spheroids	• Cost-effective • Simple protocol • High reproducibility • Allow cell–cell and cell–niche interactions • Promote cancer stem cells markers' expression • Possibility for co-cultures • Extensible in various plate formats	• Variability in size • Limited supply of oxygen and nutrients to the spheroid core
Organoids	• Tumour and patient-specific • Recapitulation of original tissue and tumour architecture and heterogeneity • Drug testing • Potential for personalized treatment	• Complicated assay • System variability • Results depend on matrix composition
3D bioprinting	• Tailored architecture • Possibility for co-cultures • Large-scale drug screening	• Cells and materials-related challenges • Specialized apparatus • Lack of vasculature • Complications with tissue functionality and maturation

on breast cancer cell fate with the use of mechanically-regulated alginate hydrogels, while Puls *et al.* [27] highlighted the importance of collagen I oligomer fibril microstructure for the induction of EMT in pancreatic ductal adenocarcinoma and presented oligomer-based matrices that could potentially serve as platforms for drug screening. Moreover, synthetic hydrogels that can be modified to bear defined characteristics including specific proteolytic sites and even to encapsulate growth factors have also gained traction. Suo *et al.* synthesized HA hydrogels with similar topography and properties as breast cancer tumours that were then used for *in vitro* and *in vivo* studies. Their results demonstrated that, compared with 2D-cultured cells, the hydrogel-cultured MCF-7 breast cancer cells better replicated the malignant phenotype, properties and expression profiles of breast cancer cells. Hence, HA hydrogels could serve as a promising matrix-mimicking platform [28].

Other synthetic hydrogel polymeric matrices, like polyethylene glycol (PEG), polyisocyanides (PIC) and polylactic acid (PA)-based, have also been proposed as platforms to study cell behaviour and therapeutic response [29,30]; among them, a multiarm PEG functionalized with collagen-like peptide hydrogel, alone and/or conjugated with peptides mimicking fibronectin and laminin, was recently used to grow human glioblastoma, rat glioma and human melanoma cells and

to access the functional properties and formation of focal adhesions (Fig. 2). Encouraging data revealed the importance of different adhesion peptide motifs in the hydrogels that correlated with changes in the behaviour of the cancer cell lines used [31].

Matrix-free culture systems

The matrix-free 3D culturing relies on the cells' self-aggregation capabilities when being grown in specialized culture settings, such as the hanging drop assay and culture in ultra-low adhesion plates (Fig. 2). These optimized cell culture settings advance the formation of cell aggregates, termed spheroids. These spheroids form more complex structures that better mimic the shape and size of cells *in vivo*, allow for enhanced cell–cell interactions, thus influencing cell behaviour and function, and provide a more physiologically relevant environment with gradients of nutrients, oxygen and signalling molecules. Particularly in the case of cancer cells, these distinct characteristics and properties make for an invaluable tool that more closely resembles tumours and their microenvironment [32,33].

The hanging drop technique drives cell aggregation and spheroid formation when cell suspension is distributed on a non-adherent surface, i.e. culture dish lid or mini-tray, and then inverted to form droplets. Alternatively, specialized hanging drop plates are also

available. Hanging drop models take advantage of the controlled and adjustable spheroid size, the inexpensive equipment needed and the large number of spheroids that can be easily produced per experiment. Moreover, ultra-low adhesion plates are specially coated culture plates that lack attachment surfaces and subsequently promote sphere formation [34,35]. Our group has recently applied such protocols for the culture of breast cancer cells in ultra-low adhesion plates, with the morphological organization of the cells being profound when compared to conventional 2D cultures. For example, in the breast cancer cell line MDA-MB-231 the cells typically appear elongated and spindle-like in the traditional 2D monolayers. As spheroids, however, they are organized as aggregates and exhibit a profound globular shape (Fig. 3 and Video S1).

Despite their success in sphere formation in a matrix-free environment, such platforms do have their limitations. To that end, new technological advances allow the development of a magnetic levitation-based platform, in which cells are mixed with magnetic nanoparticles and placed between two magnets, causing the cells to levitate, which provides an ideal microenvironment that prompts cell–cell interactions and 3D cell culture formation through the secretion of cells' own ECM [36]. Another interesting method, the hanging drop arrays, support not only the formation of uniformly sized spheroids but also drug testing upon the utilization of existing high-throughput screening instruments. Huber *et al.* [37], for example, established a hanging drop-based 3D test system for the assessment of various drugs for lung cancer and illustrated that this setup, adjusted accordingly for cell type, can be used as a drug-efficacy assay. Furthermore, Wu *et al.* cultured malignant serous effusion cells in 2D and 3D using a hanging drop method and testing them with a panel of drugs commonly used in cancer treatment. The results of the study demonstrated that the 3D cell cultures were more similar to the original patient tumours than 2D cell cultures and could be used to predict drug responses with greater accuracy, showcasing a practical alternative for drug testing in a personalized manner [38].

Latest advances in hybrid 3D culture platforms

Combining the matrix-based and matrix-free systems, hybrid approaches of incorporating spheroids into 3D biodegradable scaffolds could also prove to be a good strategy for drug screening as it significantly aids in drug response and resistance screenings that more closely resemble *in vivo* tumour growth patterns [22].
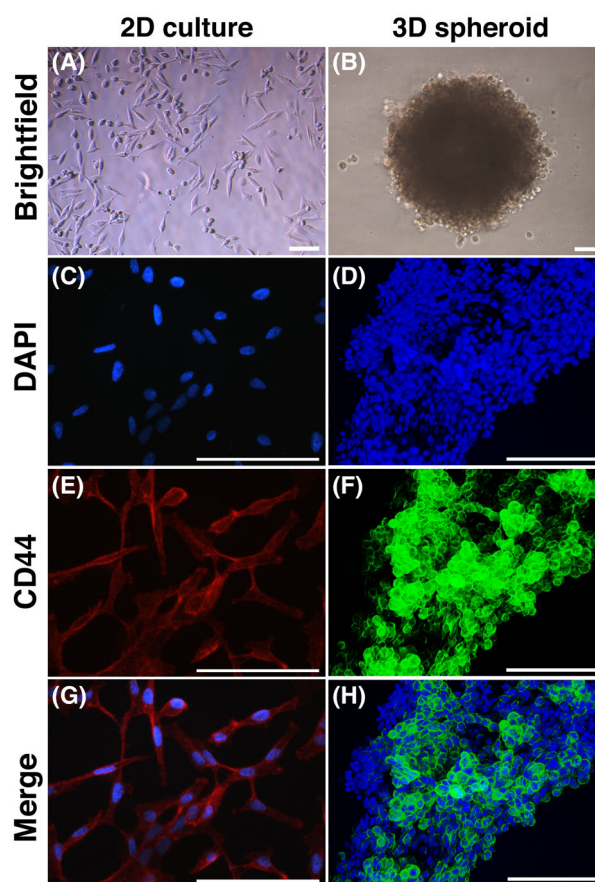

Fig. 3. Cell organization, phenotype and molecular features in conventional 2D cultures versus 3D spheroid formations. In conventional 2D cultures, MDA-MB-231 breast cancer cells exhibit their elongated and spindle-like morphology (A). Culture in ultra-low adhesion plates permits the formation of 3D spheroids where cells demonstrate a profound globular shape, organized as aggregates (B). Immunofluorescence analysis (C) and confocal microscopy (D) further highlight these differences. The expression of CD44, a major cell membrane receptor in breast cancer cells outlines individual cells, was confirmed in both cell culture platforms (E–H) demonstrating the differences in individual cell morphology. Scale bars, 100 μm. A video presentation of MDA-MB-231 cells stained for CD44 is provided in Video S1.

The most cutting-edge advances in 3D culture systems focus on organoid models (murine or patient-derived), as well as microfluidic devices and 3D bioprinted models (Fig. 2). Hans Clevers in 2009 was the first to propose a culture system, termed "organoid", where intestinal stem cells generated a self-organizing structure that resembled the normal gut epithelium [39]. Since then, various iterations of organoid culture systems have opened new avenues in cancer research and beyond [40,41]. The main advantage of organoids in general, and patient-derived organoids (PDOs) in

particular, is that they sustain the primary tumour heterogeneity making them more appropriate for the identification of biomarkers, target molecules and the verification of drug response [42–44]. Furthermore, organoids are suitable for long-term culture, cryopreservation and high-throughput screening. On the downside, organoids require careful handling and are costly models, which—even though they use a matrix scaffold—they lack in tissue components, such as vasculature or immune cells and cannot accurately mimic the primary cell-ECM and cell-stroma interactions. Also, intra-organoid variability or inconsistencies between matrix batches and starting cell lines are something that will have to be addressed and minimized for more reliable results [45]. To address some of these issues, Prince *et al.* [46] proposed the use of a nanofibrillar hydrogel (EKGel) as scaffold for breast cancer PDO. EKGel-grown PDOs presented histopathologic characteristics, expressional patterns and response to drugs similar to those of the parental tumours but also PDOs grown in basement membrane extract. Additionally, EKGel exhibits lower variability among batches along with suppressed contamination by noncancerous cells. Based on these results EKGel could be a good alternative matrix that enables inter- and intra-patient heterogeneity and has the potential to be used as a tool for personalized treatment. It is worth noting that before the development of organoids, researchers had already established another patient-derived model for cancer research, the patient-derived xenografts (PDXs). In PDXs, either cancer cell lines or biopsies from patients are transplanted into genetically modified or immunocompromised mice mainly to mimic the original human tumours and predict chemotherapy responses. However, these models fail to provide an accurate representation of the patient tumours, especially given the compromised immune response of the mice, and are time-consuming. Thus, in an era of intense effort to reduce and replace animal use organoids have offered a good alternative modelling platform. Studies that compared PDXs and PDOs recognized that PDOs can recapitulate patient heterogeneity where PDXs fail, in a shorter period of time and with lower cost [47,48]. Also, Sachs *et al.* [40] showed that the organoids were able to match the drug responses in both patients and PDXs, hence providing more evidence for the possible use of organoids not only to create biobanks but also as preclinical models to replace animals.

During the last decade, various microfluidic devices have been used as culture platforms to better imitate the functional and structural features of the actual tumour microenvironment; from tumour-circulatory system interactions to crosstalk between different cell types [49–51]. Microfluidics have been proven especially helpful for the study of cancer metastasis and angiogenesis and can also potentially harbour circulating tumour cells culture and drug screening [52].

3D bioprinting is a novel tissue engineering technology that offers the reconstruction of tissues and organs with the hierarchical architecture of their native counterparts and could be beneficial for our perception of cancer pathobiology and metastasis as well as the development of new cancer therapeutics. Dankó *et al.* [53], for instance, fabricated an alginate-based hydrogel bioink for 3D bioprinting that mimics the *in vivo* microenvironment of breast cancer for drug screening and metabolic targeting between 2D and 3D cultured cells, as well as xenografts, with the 3D model providing a closer representation of the *in vivo* results. Additionally, Almela *et al.* [54] managed to produce a 3D-printed cancer model for oral squamous cell carcinoma that could replace *in vivo* models for the assessment of diagnostic and other approaches. On the other hand, Mondal *et al.* [55], combined 3D extrusion bioprinting with synthetic hydrogels to develop a sodium alginate/gelatin scaffold where they studied the interplay between non-small-cell lung cancer, PDXs and CAFs in co-culture spheroids. These preliminary studies lay the ground for more in-depth studies that will optimize this technology and allow for wider application as a substitute for *in vivo* studies.

Conclusions

Studies on bioscaffolds for the development of advanced 3D culture systems that mimic the *in vivo* models have grown exponentially in recent years, providing great prospects for a wide range of applications in a variety of diseases, including cancer. The main challenge right now remains the creation of novel platforms, including matrix-free and matrix-based 3D culturing, 3D bioprinting and PDOs, which recapitulate microenvironmental cues that mirror the *in vivo* pathology of the disease. In this context, combining knowledge from bioengineering with the availability of multi-omics data could offer revolutionary possibilities in understanding the role of genetic/epigenetic programs governing the tumour matrisome and allow the design of biomimetic models through targeting specific ECM biomarkers [56]. The stochastic evaluation of all the microenvironmental interactions will shed light on the high-importance factors that drive specific phenotypes and in turn will allow the design of scaffolds that most accurately mimic these conditions.

Materials and methods

Cell culture and imaging

For the 2D cultures in Fig. 3, MDA-MB-231 breast cancer cells were seeded on glass coverslips in 24-well plate at a density of 5×10^4 cells per well. For the 3D cultures in Fig. 3, the cells were grown at a density of 10^4 cells per well in ultra-low adhesion 96-well plates (SPL Life Sciences, Pocheon-si, South Korea). Brightfield images of the live cells were obtained through a $10\times$ objective lens of an optical microscope (OLYMPUS CKX41, Waltham, MA, USA) equipped with a digital camera (QImaging Micro-Publisher 3.3RTV, Adept Turnkey, Perth, Australia). The cells were then fixed in 4% PFA and washed with PBS. Subsequently, the cells were blocked with 4% BSA in PBS/Triton-X 0.1% for 2 h and were stained overnight at 4 °C with the primary antibody against CD44 (Hermes-3; Abcam, Cambridge, UK) using a 1:500 dilution. Following PBS washes, the 2D coverslips were incubated with the secondary antibody (anti-mouse Alexa Fluor-594, 1:500; Biotium, Fremont, CA, USA) for 2 h and were mounted on microscope slides with DAPI. Similarly, the 3D spheroids were incubated with anti-mouse Alexa Fluor-488 (1:500; Biotium) for 2 h before being carefully transferred on microscope slides and mounted with DAPI under a coverslip. The immunofluorescence imaging was performed on a Leica (Wetzlar, Germany) TCS SP8 confocal microscope using the $60\times$ lens.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

KK, ZP and NKK performed study conception and design. KK, CK and ZP performed relevant literature review and data curation and wrote the first draft of the manuscript. ZP, CK and NKK performed study design and analysis. ZP and CK performed figure preparation. KK performed table preparation. All authors commented on previous versions of the manuscript and performed revisions. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Video S1. 3D confocal imaging of MDA-MB-231 breast cancer cells forming a spheroid. The nuclei (blue) and the cell membrane receptor CD44 (green) highlight their profound globular shape and organization as aggregates, which significantly differs from their usual characteristics in traditional 2D cultures.