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Three-Dimensional Cell Culture Models for Biomarker Discoveries and Cancer Research

Caicedo-Carvajal CE[1], Liu Q[1], Goy A[2], Pecora A[2] and Suh KS[2]*

[1]3D Biotek LLC, 675 US Highway 1, North Brunswick, NJ 08902, USA
[2]John Theurer Cancer Center, Hackensack University Medical Center, Hackensack, NJ 07601, USA

Abstract

Novel biomarker discovery requires high quality biospecimens and proper maintenance of cell phenotype. However, patient samples have reduced availability rendering not practical for large-scale drug screening and biomarker discovery. This impracticality has stimulated the use of cell lines in many "–omics" research studies to discover biomarkers. In addition, cells in these *in vitro* models are typically grown on 2-dimensional (2D) culture dishes, which is obviously different from native *in vivo* microenvironments. Data extraction from cells grown in 2D has less relevance, and thus, the biomarkers derived from studies using 2D platforms will likely have less clinical value. Thus, implementation of *in vitro* models that take into account primary patient samples and *in vivo*-like factor represent a paradigm shift in cancer biomarker discovery. This review emphasizes on current 3D cell culture platforms used to recreate *in vivo* conditions and their ability to adapt towards demanding conditions of biological relevance.

Keywords: 3D Cell culture; Novel biomarker discovery; Personalized medicine; *In vitro* 3D Cell-based models; Targeted therapeutics

Overview

Since the discovery of the cell as the basic unit of tissues, cell culture has been traditionally defined by simple approximations. The most common are two-dimensional (2D) cell culture and separating disease cells from their native microenvironment. Because these limitations result in challenges for the discovery of clinically relevant biomarkers, many 3-dimensional (3D) cell culture methods have been introduced over the past decades. Models that have recently been used in tissue engineering include 3D geometry and cell co-culture to better represent tissue homeostasis in *in vitro* settings [1]. Adopting the success of Tissue Engineering models, the same principles can also be used for biomarker discovery projects for cancer. As mounting evidence indicates that gene expression and signaling pathways of tumor tissues depend on context or their native microenvironment, these 3D co-culture models more closely resemble cancers [2]. In the tumor microenvironment, normal and cancer cells interact in a 3D setting and influence each other to positively enhance oncogenic potential [3]. For example, during cancer progression and metastasis, tumor cells up regulate oncogenes to increase proliferation while actively modify their cellular microenvironment to control angiogenesis, extracellular matrix (ECM) stiffness, proliferation, and oxygen levels [4-6]. This highly orchestrated sequence of events defines the innate plasticity of cancer cells that allows them to exert control at molecular and tissue levels to maintain a malignant phenotype [7-9]. Clearly, the conditions that surround tumor cells must be replicated in an *in vitro* setting to resemble the tumor microenvironment, and the traditional 2D tumor cell culture method is an oversimplified *in vitro* cell-based model that cannot recreate the environment of cancer homeostasis [10]. As a consequence, biomarkers identified in 2D culture often lack critical *in vivo* signatures, whereas biomarkers identified in 3D culture are more likely to ultimately enhance our understanding of tumor biology, anti-cancer drug development, and future personalized medicine applications [11,12].

Three-dimensional tissue culture has excellent potential for recreating cancer models. Several studies, from tumor organoids to matrix-based cell culture, have shown that cancer cells grown in 3D models are more phenotypically stable and closer to the parent cell line phenotype than cancer cells grown as 2D monolayer [13]. In addition, drug resistance in cancer cells grown in 3D models is consistent with *in vivo* resistance, whereas similar cell types have been shown to have drug sensitivity when grown in 2D models [6,14,15], suggesting that 3D culture platforms are required to discover biomarkers that are disease relevant and related to clinical phenotypes of the tumor. There is strong evidence that 3D growth positively recreates cancer behavior and can include other factors similar to *in vivo* conditions, including animal-based pre-clinical models that might lead to advancements towards personalized medicine [16,17].

Figure 1 provides a conceptual representation of the progression of cell-based models and the complexity associated with recreating *in vivo*-like conditions, such as 3D geometry, cell co-culture, and perfusion. To correctly reproduce *in vivo* features, all of these factors must come together, but they often need to be added to *in vitro* models in a step-wise manner. If the 3D models differ from the *in vivo* tumor microenvironment, any biomarkers that are identified in that setting will not reflect the "true" disease. In the diagram, each incremental step causes an increase in model complexity. At the same time, the increase in complexity leads to simulating more *in vivo*-like cell culture conditions, which is particularly important for discovering novel biomarkers. For example, the addition of dimensionality to current cell culture models has brought about the implementation of other important *in vivo* factors to established, 3D cell-based platforms, recreating a better approximation to current animal models [17,18]. However, depending on the 3D methodology used to recreate *in vivo*-like models, there is an equal increase in the level of experimental complexity upon implementation of *in vivo* factors. This may represent a turning point when choosing a specific 3D cell culture platform that closely resembles *in vivo* conditions for biomarker discovery projects.

***Corresponding author:** K Stephen Suh, The Genomics and Biomarkers Program, John Theurer Cancer Center, Hackensack University Medical Center, Hackensack, NJ 07601, USA, Email: ksuh@humed.com

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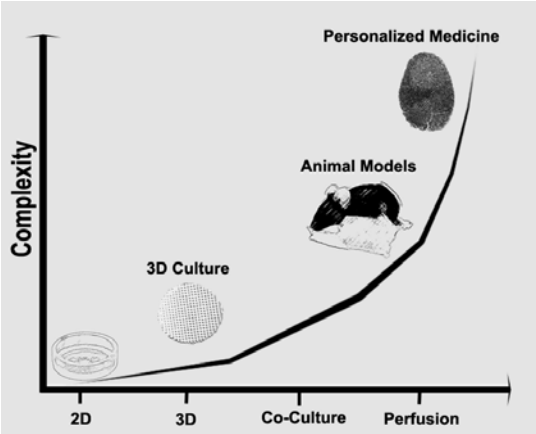

Figure 1: Conceptual Progression of *in vitro* Cell-Based Models. Three-dimensional geometry represents the first step towards complex *in vitro* cell-based models. Implementation of other factors, as shown on the x-axis, would increase the complexity of *in vitro* models. This may represent the road to integrated *in vitro* cell-based technologies, an alternative to animal models, and their potential use with primary patient samples for biomarker drug screening.

3D Cell Culture Platforms

As a result of robust differences in cell phenotypes and molecular signatures when cells are grown in 3D versus 2D culture, many 3D cell-based platforms are being rapidly developed. These 3D platforms vary in material properties and material processing, and their structures include reconstituted ECM, synthetic hydrogels, porous polymer scaffolds, and nano-topography. Even though 3D cell behavior is easily reproduced across the spectrum of available 3D cell-based platforms, there is still a need to define 3D cell-based platforms as an integrative tool for direct assessment of native cancer microenvironment for biomarker studies. Cell culture in three dimensions must replicate complex heterogeneous cell mixtures, perfusion, and the dimensionality of *in vivo* disease environment.

The diverse examples of available 3D cell culture platforms can be viewed as converging methodologies with the ability to include *in vivo*-like conditions such as porous architecture, enabling cell self-assembly, perfusion, and co-culture with multiple other cell types. As the 3D conditions are constructed, the technologies should diverge from the presence of animal-derived trace elements such as morphogens and reconstituted extracellular matrix, as a result of primary patient tissue biopsies. The purpose of this review is not to minimize the importance of other technologies, but rather to define the most biologically relevant technologies for biomarker discovery that can contribute to future developments in personalized medicine. In figure 2, we provide a basic representation of cancer tissue architecture. This architecture is composed of a heterogeneous ECM; several cell types such as normal cells, cancer cells, and cell stroma, all communicating through normal and defective signaling profiles [16,19-21]. The challenge is to provide a 3D platform that allows primary cells from tissue biopsies to recreate the closest *in vivo* approximation of cancer ECM and morphogen signaling patterns [17]. For the purpose of illustrating how 3D conditions can support biomarker discovery projects, several 3D cell culture technologies will be described while emphasizing that the challenges of constructing an ideal 3D culture model are usually better approximated with synergistic approaches rather than absolute methodologies.

Multicellular Spheroid-Based Platform

Multicellular spheroids are the closest *in vivo* representation of *in vitro* tissue self-assembly that is driven by active cell-cell and cell-ECM interactions [22-25]. The balance of these adhesive interactions has been found to control normal morphogenesis in healthy tissues [26,27] and during cancer metastasis [28,29]. Thus, it is sometimes appropriate to consider spheroids as 3D culture models for cancer biomarker projects. As a 3D model, spheroids have become a useful tool for recreating the 3D tumor microenvironment in the absence of external scaffolding [30].

Cell spheroids are usually generated using the hanging drop method [31], by shaking cell suspensions with continuous motion [32], or by the traditional soft-agar method [33]. All of these methods partly represent 3D conditions *in vivo* because they allow anchorage-independent cellular aggregation. In the case of the hanging drop method, 10- to 15–μλ aliquots of cell suspension are placed on the underside of a polystyrene dish where they hang as drops from the top of the plate. Over several hours and days, cells coalesce to the bottom of the drop at the liquid-air interface, forming cellular aggregates [34]. Current technologies have optimized spheroid formation on 96-plate formats, minimizing the processing time for drug screening efforts. The shaking flask is a simple method that facilitates the formation of multicellular spheroids from cell suspensions shaken at specific rpm values under regular cell culture conditions [35]. Lastly, soft-agar applications require mixing cells in agar at 37-38°C to make a single layer of agar-containing cells, followed by a second overlaying agarose layer containing nutrients for long-term culture [33].

There are several advantages to such self-assembled cellular systems. Multicellular spheroids are not constrained to external material properties, allowing cells to develop intrinsic material properties. In particular, cancer has been found to express tissue-specific stiffness associated with its malignancy, offering more drug screening targets [36,37]. One of the major advantages of cancer spheroids is the recreation of regions of differential growth/metabolism such as highly dividing cells at the surface, quiescent cells in the middle, and hypoxic necrotic cells at the center [38]. Regions of differential growth are of particular interest because anti-cancer drug resistance is readily

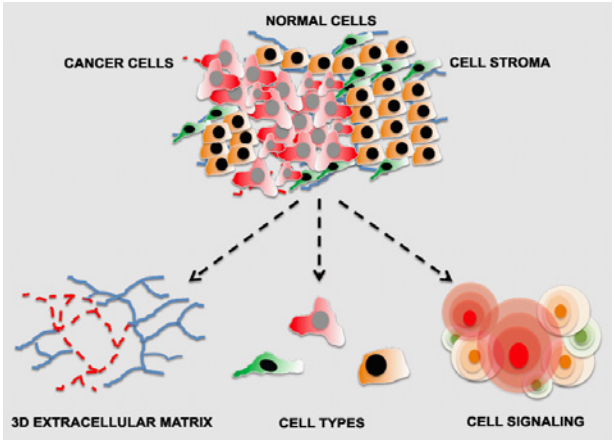

Figure 2: The Three Basic Components of Cancer Tissues. Healthy and diseased tissues share the same components: extracellular matrix, cells, and cell signaling gradients. However, cancer tissue architecture is far more complex than that of normal tissues. The cancer microenvironment is characterized by the heterogeneity of its single components, from the ECM to cell signaling events. Thus, each component of the cancer architecture is equally important for targeted therapeutic.

expressed in areas of poor diffusion and reduced vascularization [39,40]. Drug resistance is a multi-factorial event involving a combination of receptor-mediated pathways and cellular architecture [41]. Thus, cell spheroids have been used to test anti-cancer drug analogs and to elucidate drug resistance mechanisms *in vitro*, such as angiogenesis-blocking therapies directed to the vascular endothelial growth factor (VEGF) pathway and hypoxia-induced chemoresistance due to upregulation of HIF-1α and HIF-2α [39,40,42].

Potential Pitfalls for Screening Applications

On the other hand, handling multicellular spheroids may become cumbersome when dealing with large numbers in small platform formats required for studying biomarkers to drug responses. For these types of studies, many smaller platforms must be constructed to test multiple types of drugs. In addition, the soft-agar method requires long culture times (weeks) to generate spheroids, and cell embedding may become an issue due to the temperature ranges used to mix cells and agar. In fact, most of the issues with multicellular spheroid-based platforms are not related to their ability to recreate cell behavior, but rather to the processing of spheroids for large-scale screening of complex cell mixtures such as patient biopsies and tissue homogenates. Thus, this method is appropriate for cell lines or a single tissue type that can generate a large number of cells, such as recreating liver tissue equivalents. As we further develop into the following sections, polymer-based technologies are leading the way in the incorporation of microarrays for handling/recreating multiple cellular spheroids [43,44], offering advantages such as decreased processing and the maintenance of native multicellular spheroid architecture for biomarker studies and, to a larger extent, for drug screening applications.

Gel-Based 3D Cell Culture Platforms

Cells *in vivo* are either embedded within the ECM or in contact with the basement membrane (BM) during maintenance of normal basal epithelial polarity [45], and this is a crucial component to consider when constructing a 3D culture model. For proper biomarker discovery, the cellular surroundings and the matrix components must be taken into account because they play vital roles in the tumor microenvironment. The ECM makes up a large fraction of the total volume of a tissue, and its complexity and gel-like composition vary according to the type of cells that are actively building their microenvironment, and thus defining specific cellular continuums. For example, during development chondrocytes secrete a variety of ECM components such as keratan sulfate, chondroitin sulfate, and glycosaminoglycans. This type of ECM defines the specific tissue function and mechanics of native cartilage [46]. On the other hand, cells comprising lung tissue, arteries, and skin generate elastin, a different ECM protein that confers other mechanical properties such as compliance to lungs, arteries, and skin tissues [47]. Thus, each tumor microenvironment must be replicated as closely as possible in the 3D culture model to correctly represent the disease environment.

Animal-Based 3D Gel Platform

Even though the ECM defines healthy tissue structure, tumor-mediated ECM reorganization has been linked to cancer prognosis and progression [48-50], defining the need to recreate the native gel-like state of tumor cells and to better assesses oncogenic potential. For example, cancer metastasis and angiogenesis have been extensively studied using a BM derivative (Matrigel) from the Engebreth-Holm-Swarm (EHS) mouse sarcoma because it closely resembles the material properties of laminin- and collagen-rich microenvironments [51,52]. As an alternative to animal-based gels, other forms of polymer-based gels have also been developed to recreate native cell microenvironments [53]. Hydrogels are hydrophilic polymeric networks able to exponentially take up water, thus recreating the naturally hydrated environment of living tissues. Hydrogels can be tailored from either synthetic materials or from a combination of bioactive and synthetic materials [54]. As a result of their versatility, hydrogel matrices are widely used to study specific cellular behavior/responses related to cellular maintenance, migration, and material stiffness [55-57]. Particularly, synthetic hydrogels have been widely used in tissue engineering, with direct application in a wide range of regenerative approaches [54,58].

Potential Pitfalls for Screening Applications

Matrigel has shown *in vitro* and *in vivo* bioactivity, for example, generation of complex epithelial-like structures, chemoinvasion, and angiogenesis, as a direct result of its ECM and morphogen composition [59-62]. However, Matrigel's complex and variable morphogen composition can increase the difficulty of interpreting experimental results and the level of false positives and false negatives in drug screening studies [63,64]. In perspective, the use of Matrigel in 3D cell-based drug screening models goes against current animal-free approaches to cell culture. Following the tenet that more defined and less complex media formulations result in the lowest background during synthetic and protein-based drug screening, the same rationale should be adopted in 3D tissue-based models.

Animal-Free 3D Gel Platform

As a consequence of the intrinsic complex microenvironment of animal-derived matrices, polymer-based matrices are gaining interest for cell-based applications. One of the major advantages of hydrogels is the versatility to deliver potential drug analogs via controlled-release drug devices. For example, hydrogels can be used as wound dressings, allowing the incorporation of various growth factors to increase the rate of healing in skin grafts or angiogenic factors for post-infarction tissue repair [65,66]. In addition to controlled delivery of biologics, hydrogels are also designed for the delivery of synthetic drug analogs for cancer treatment. In particular, controlling the hydrogel-drug interaction is an important concept as drug and hydrogel chemistries may be tailored to achieve desirable release profiles during drug treatment [67-69]. This

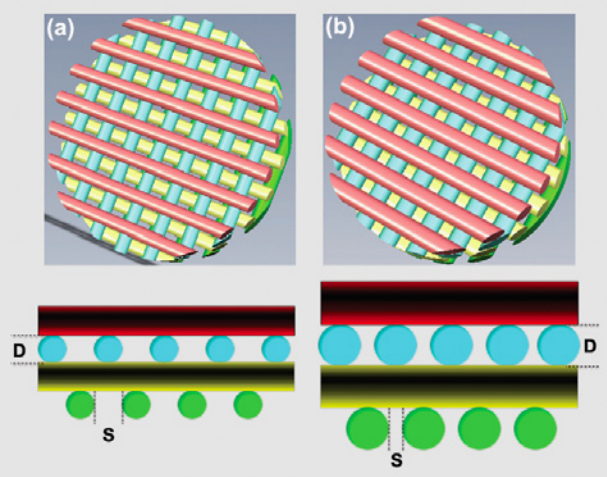

Figure 3: CAD Images of Freeform 3D Scaffolds. 3D scaffolds can be designed for controlled dimensions of fiber diameter and fiber-to-fiber spacing, maintaining a characteristic open-pore 3D structure. Images (a-b) show examples of variation in fiber diameter (D) and fiber-to-fiber spacing (S).

offers an advantage given that screening for synthetic drugs usually yields analogs with variable chemistries, making hydrogels useful in tailored drug-release devices.

As a result of hydrogel versatility, hydrogels can be fabricated into porous structures with variable stiffness [70]. Porosity is a feature of great importance for cellular growth leading to tissue formation, further addressed in detail under "*Polymer Deposition Platforms: From Nano- to Micron-Scale Fibers*". Simulating stiffness is an important material property found in normal and diseased tissues [71]. For example, agarose and sucrose have been used to generate porous hydrogels that support the growth of hepatocarcinoma cells. These hydrogels show an inverse relationship between porosity and compressive modulus ranging from 5% to 45% porosity and 140-KPa to 20-KPa compressive modulus. However, hepatocyte viability varies with porosity and distance from the surface of the hydrogel construct [70]. From the perspective of cancer malignancy, cross-linked collagen 3D hydrogel constructs have shown a relationship between stiffness and cancer phenotype. Hepatocarcinoma spheroids interacting in softer constructs showed an increase in malignancy, whereas stiffer hydrogels caused hepatocarcinoma spheroid compaction and suppressed malignancy [72]. Similar behavior has been observed using *in vitro* glioblastoma models, correlating with increased malignancy within brain tissue [73,74].

Adaptability towards Screening Applications

Although hydrogels can be used to recreate the 3D hydrated architecture of native tissues, further applications of the technology are becoming available in the field of cell-based drug screening: for example, generation of drug-eluting porous hydrogels models for *in situ* drug release and fabrication of hydrogels into millimeter-scale networks to accommodate large numbers of micron-scale 3D microenvironments [43,75]. Using fast-precision fabrication such as micro-fabrication, micro-contact printing, polymer microinjection, and microbubble technologies, controlled-geometry hydrogel constructs have been tailored to generate multi-cellular spheroid arrays. Particularly, small-scale chips can be tailored to have multiple micro-wells connected through micro-channels, favoring spheroid formation and perfusion of primary hepatocytes and HepG2 hepatocarcinoma cells for drug screening applications [43,76]. Microinjection of cell-polymer suspensions into collagen gels is another example of controlled small-scale optimization. Microinjection allows control of spheroid size, because exact volumes of cell suspension-polymer can be dispensed into the gel template and the polymeric vehicle can be optimized to speed-up cellular aggregation [77], increasing both model precision and processing time. These examples represent synergistic benefits to maintaining 3D cellular geometry while providing large-scale processing at the micron-scale level.

Polymer Deposition Platforms: From Nano- to Micron-Scale Fibers

Polymer-based platforms for 3D culture provide an excellent condition for biomarker studies. Polymers are formed of repetitive units assembled into long chains. The chemical versatility of polymers derives from the fact that polymer units can differ with respect to side chain modifications, unit chemistry, and type of unit bond chemistry. Naturally occurring polymers are ubiquitous in living organisms as part of structural components such as collagen, keratin, cellulose, and chitin. Synthetic chemistry has allowed the generation of libraries of synthetic polymers with the ability to be modified, offering a versatile product for applications in medicine and tissue engineering.

Polymeric materials can be processed into various shapes and geometric configurations, offering an advantage for applications in tissue engineering and cancer biology. In terms of 3D geometry, polymer deposition geometries range from nano-scale to micro-scale, both benefiting cellular growth and maintenance [78]. Depending on the application, polymers can be fabricated using a variety of physical processes such as gas foaming, sintering, solvent casting, microencapsulation, electro spinning, patterning, and solid freeform deposition. The common feature of these polymer-processing methods is the generation of 3D architectures with surface area and void volume (interstitial space). Biologically, surface area allows initial cellular attachment and the void space defines the future cellular microenvironment, which is composed of different cell types, native ECM proteins, and signaling gradients (Figure 2). Traditionally, micro-porous scaffolds have been generated by gas foaming, phase separation, and salt/sucrose polymer blends [79,80]. Here, the porous structure is defined by the size and distribution of bubbles and salt/sucrose crystals in the foam phase and polymer melts, respectively. Although these processes generate micron-scale porous architectures, the random nature of the architecture leads to reduced pore-to-pore interconnectivity, creating potential issues such as uneven cell distribution/infiltration and suboptimal exchange of nutrient/waste cellular products.

Micron-Scale 3D Polymer Fiber Deposition

Fiber deposition technologies are adapting towards controlled 3D polymer deposition to better define pore size and, most importantly, to maintain pore-to-pore interconnectivity [81,82]. From the available polymer deposition technologies and current adaptation towards controlled 3D geometries, rapid polymer prototyping technologies have shown great promise for controlling 3D pore geometry because polymer fibers are laid down using a programmable coordinate system [83-86]. Using a computer interface, parameters such as fiber directionality, fiber diameter, and fiber-to-fiber spacing can be controlled with high accuracy. Figure 3 illustrates the geometric versatility of prototyping technologies such as freeform scaffold fabrication (FFF). Three-dimensional complexity depends on pore size, pore-pore interconnectivity, and fiber directionality. In Figure 3a and 3b, fibers are laid orthogonal and offset to each other, creating a wavy-like pattern of pores within the four-layered structure. Another level of complexity is the ability to control the pore size upon variation of fiber diameter (D) and fiber-fiber spacing (S), and the degree of pore connectivity upon variation of fiber diameter (D) at constant fiber-to-fiber spacing (S) (scaffold cross-sectional areas shown at the bottom). This versatility and geometric control has proven to be beneficial in the generation of cell-based models.

The ability to change fiber dimension and fiber spacing defines the advantages of precision deposition porous structures. For example, Figure 4 shows the relationship between polymer fiber diameter and pore size, a direct result of polymer fiber deposition. The red, blue, and green dotted lines represent ranges of fiber diameter and respective pore sizes for three different electrospun polymers [87]. The black dotted line represents ranges of fiber and pore sizes from solid freeform deposition of poly-e-caprolactone (PCL) and polystyrene (PS) [78,83,86]. Independent of polymer chemical composition and polymer deposition process, pore size has been found to increase in proportion to increasing fiber diameter [88]. The importance of pore size in reference to the range of cell diameter is related to the ability of passive cell suspensions and active cell migration to infiltrate the internal porous structures. For example, cellular infiltration diminishes as pores approach nano-scale sizes. On the other hand, better cell

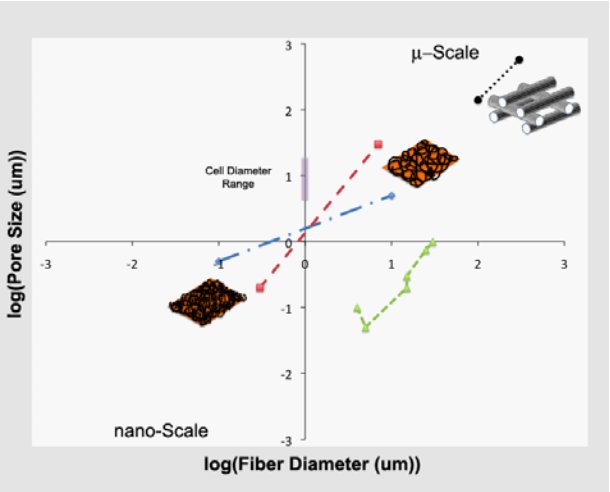

Figure 4: The Effect of Fiber Diameter and Pore Size on 3D Porous Architecture. We plotted the range of fibers and their respective pore sizes for different electrospun fibers: red dotted line (electrospun copolyesters); blue dotted line (experimental and theoretical data); and green dotted line (electrospun polystyrene). The black dotted line represents the range of porous scaffolds made using free-formed polymer deposition. Although the ranges differ, in each case fiber diameter is proportional to pore size.

infiltration is achieved as pore size increases, such as with micron-scale pore sizes [89]. Thus, the dynamic range of 3D architectures derived from varying fiber and pore sizes offers a wide range of applications for integrated 3D disease models.

Nano-Scale 3D Polymer Fiber Deposition

At the nano-scale level, spectrospun fibers have been shown to modulate the commitment of embryonic and mesenchymal stem cells to osteoblastic and chondrogenic lineages in the absence of osteogenic- and chondrogenic-inducing factors [78,90]. These effects have been associated with the organization of cellular adhesion in response to nano-scale topography, resembling native ECM roughness [78,91,92]. Although cellular processes such as lamellapodia and filapodia penetrate the nanofiber structure at pore sizes greater than 900 nm [78], efficient cell infiltration is not possible with pores smaller than 10 μμ [89].

In addition to tissue engineering applications (i.e., nano-scale control of stem cell fate) nanofiber constructs have been used as drug release devices for cancer therapeutics. One particular advantage is the ability to blend therapeutic agents with biodegradable polymers, thus controlling *in vitro* drug release and activity [93-95]. These types of polymer-based 3D cultures can be subcutaneously inserted into animal models to generate better xenograft tumor models and discover biomarkers that are more relevant to human cancer. Thus, such 3D platforms can be translated from pure 3D *in vitro* culture models to 3D *in vivo* xenograft models that better represent the disease. Other applications of nanofibers in cancer research are gaining interest. For example, Fischer et al. [96] showed that coated electrospun PCL nanofibers with fibrotic and normal lung tissue extracts recreate the idiopathic pulmonary fibrosis (IPF) microenvironment and have an inductive effect on bone marrow derived cells. IPF is characterized by an increase in the number of myofibroblasts and extensive ECM deposition, thus defining potential therapeutic targets. It was demonstrated that bone marrow cells adhered differently, secreted extensive ECM, and showed upregulation of myofibroblastic gene profiles when grown on fibrotic-coated nanofiber mats. The same effect was achieved by increasing nanofiber stiffness in the absence of fibrotic lung extract [96].

Another application of aligned nanofiber architecture is to quantify malignant cell migration for potential targets of metastatic cell pathways. Gliomas seeded on nanofibers resembling the neural topography showed *in vivo*-like properties such as elongated morphology and upregulation of glioma STAT3 signaling. Consequentially, inhibitors of STAT3 cause reduction of glioma migration at sub-toxic inhibitor concentrations. These behaviors were totally opposite to those of gliomas seeded on 2D surfaces and random nanofiber architecture [97]. Nano- to sub-micron scale fibers offer distinct advantages as these topographies have the ability to mimic the native ECM roughness. They also offer advantages in targeted therapeutics when there is a clear understanding of the effect of nano-topography on cellular behavior. As nano-topography can direct normal cells into differentiation lineages, there is also the possibility to direct defective cells away from their true nature in the case of certain types of cancers.

Adaptability towards Screening Applications

In contrast to nano-scale pore and fiber structures, micron-scale architecture offers other benefits suited for maintenance of cell function, from the most basic concept such as helping cells to infiltrate the internal 3D geometry by means of passive diffusion of cell suspensions, active cell migration, and maintenance of cell function [98-100]. In addition, these micron-scale 3D scaffolds can be miniaturized to fit small wells for screening purposes. Both the manufacturing and open-pore 3D structure defines the application of FFF scaffolds as a tool to recreate primary tissue models, e.g. tissue biopsies and needle aspirates, for biomarker screening and personalized medicine screening platforms. The following publications outline the strengths of FFF scaffolds for recreation of realistic screening *in vitro* models.

Several publications have reported the ability of freeform polymer deposition to aid cell infiltration and proliferation in 3D *in vitro* models. To elucidate some of them, Dainiak et al. [53] used systematic screening of several 3D scaffolds made with different polymer processes. They wanted to determine the effect of different 3D architectures on primary human bone stroma cells (hBMSCs), during differentiation and proliferation. In the study it was found that hBMSCs differentiated into osteoblastic lineages when after adhering to electrospun PCL nanofibers (300-900 nm) in the absence of osteogenic factors after 50 days in culture. As expected micron-scale 3D scaffolds did not cause induction of cells into osteogenic lineages. However, there were marked differences in the proliferation of hBMSCs as a function of type of 3D geometry. For example, salt-leached and gas-foamed PCL scaffolds with porosities greater than 90% and pore sizes ranging from 300 μμ το <100 μμ had less proliferation and evenly distributed hBMSCs than FFF PCL scaffolds with 65% porosity and 580nm pore size. FFF PCL scaffolds showed superior proliferation of hBMSCs both in basal and differentiation media at 1, 3, and 7 weeks in culture [78]. As previously described in figure 4, there is a dynamic range of cellular infiltration, both active and passive, that re dictated by the geometry of the 3D scaffold i.e. larger pore size and pore-to-pore interconnectivity. This demonstrates that random pore formation yields highly porous structures, but does not promote pore interconnectivity, an important geometric feature that enhances cellular infiltration and growth progression. As in the case of hBMSCs, the choice of 3D architecture represents a critical issue when culturing primary tumor cells requiring optimum growth conditions.

Besides using 3D scaffolds for Tissue Engineering and stem cell

differentiation, precision deposition 3D scaffolds have been used to recreate complex co-culture models such as lymphoma and stroma. Stroma, an ubiquitous compartment, has been found to aid in the progression of cancer [101]. The same stroma covers the conduits of secreting glands and mediates contact during paracrine-mediated bone marrow and lymphocyte maturation [102-104]. This represents a complex condition, as stroma defines the scaffolding of a tissue and blood cells are mostly in suspension. The same conditions are found in blood cancers. Lymphoma is a type of blood cancer originating in the lymphatic tissue, which eventually adapts to proliferate in the blood compartment thus increasing its malignancy. Using FFF scaffolds, it was demonstrated that at day 7 fewer than 1% lymphoma cells mixed with stroma were able to amplify 200-fold, representing a 3.2-fold higher proliferative rate than 1% lymphoma: stroma co-cultures on 2D model and an 8-fold increase than lymphoma grown in suspension [86]. This finding further validates, besides maintenance of optimum growth conditions in hBMSCs, the versatility of the scaffold as a tool for complex cellular mixtures such as tissue biopsies and needle aspirates. This type of amplification method can be applied to primary cancer cells derived from a limited amount of patient samples to study disease biomarkers and extended to potential use in personalized drug screening applications.

Summary

In the current era, striving toward personalized medicine and targeted therapy, creating the most appropriate *in vitro* model that closely mimics the *in vivo* tumor microenvironment is one of the most important topics to address scientifically. Currently there is a mixed of traditional 3D platforms and emerging technologies relaying heavily on the advantage of polymer processing to recreate porous structures for cell maintenance. It is an easy task to grow cells in 3D; another is to generate models that closely resemble *in vivo* conditions. In addition to current effort to switch from animal-free models and implement primary patient samples for personalized drug screening and biomarker discovery.

Currently, there are two important needs to be met during cell-based screening processes: i) the ability to facilitate and support cellular growth of heterogeneous cell mixtures from patients, and ii) the degree of adaptability of the 3D platform towards high throughput screening. In addition, current 3D cell-based models rely on cancer cell lines to identify basic biomarkers, minimizing the purpose of 3D platforms. This is consequential effect of patient samples limited availability, especially primary tumor cells. Thus, defying the need for 3D platforms able to support cell growth and scalable towards small-scale formats. Three-dimensional culture models that can amplify primary cancer cells within a short period of time while maintaining parental molecular signatures represent a promising platform. These models must include multiple cell types that are present in the tumor microenvironment and may require extracellular factors different from those used in 2D culture models in order to maintain the proper 3D environment to correctly represent the disease. We still await 3D methods that can better represent the tumor microenvironment, but with the combination of various 3D platforms currently available we hope to discover clinically relevant biomarkers that can be applied toward personalized and targeted therapies for optimal cancer care.

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