Translational Oncology
www.transonc.com

Volume 7 Number 6 December 2014 pp. 657–664 657

Personalized *In Vitro* Cancer Modeling — Fantasy or Reality?

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Abstract

With greater technological advancements and understanding of pathophysiology, "personalized medicine" has become a more realistic goal. In the field of cancer, personalized medicine is the ultimate objective, as each cancer is unique and each tumor is heterogeneous. For many decades, researchers have relied upon studying the histopathology of tumors in the hope that it would provide clues to understanding the pathophysiology of cancer. Current preclinical research relies heavily upon two-dimensional culture models. However, these models have had limited success in recreating the complex interactions between cancer cells and the stroma environment *in vivo*. Thus, there is increasing impetus to shift to three-dimensional models, which more accurately reflect this phenomenon. With a more accurate *in vitro* tumor model, drug sensitivity can be tested to determine the best treatment option based on the tumor characteristics. Many methods have been developed to create tumor models or "tumoroids," each with its advantages and limitations. One significant problem faced is the replication of angiogenesis that is characteristic of tumors *in vivo*. Nonetheless, if three-dimensional models could be standardized and implemented as a preclinical research tool for therapeutic testing, we would be taking a step towards making personalized cancer medicine a reality.

Translational Oncology (2014) 7, 657–664

Introduction

Cancer was responsible for 1.23 million deaths within the European Union in 2008 [1]. In the United Kingdom, the incidence of all types of cancer was over 520 cases per 100,000 people, with the four most common cancers being breast, lung, colorectal, and prostate [2]. The health care cost of cancer within the European Union has been estimated at just under £44 billion. This is only around 40% of the total cost of cancer to society, which takes into account productivity loss among other factors [1]. As a consequence, a vast sum of money is invested into producing cancer treatments, which currently only produce around a 45% 10-year survival rate [3]. As such, the production of highly effective cancer treatment is a vital area of research. In order to test drug efficacy and the specific resistance of cancers to certain drugs, there must be new methods of producing pertinent *in vitro* representations of solid tumors very similar to those that occur *in vivo*. (See Table 1).

Two-dimensional (2D) monolayers remain the standard for cancer drug discovery, even though 2D monolayers are unable to replicate the complicated environment and mechanisms of a solid tumor and its growth [4,5]. The production of three-dimensional (3D) *in vitro*

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Received 7 August 2014; Revised 6 October 2014; Accepted 13 October 2014

1936-5233/14
http://dx.doi.org/10.1016/j.tranon.2014.10.006

Table 1. Summary of Advantages and Disadvantages of Various Techniques Used to Engineer 3D Tumor Models

	Advantages	Disadvantages
Cellular spheroid a. Rotary cell culture system b. Hanging drop plate c. Hanging drop array d. Microarray e. Collagen-implanted spheroids	• Produces a large number of spheroids • Consistent sizes • Does not require external scaffold • Easily applied to high-throughput screens • Spherical shape enables modeling of tumor growth and invasive processes [13]	• Complexity needs to be improved to better mimic *in vivo* tumors • TM: not all cell types and ECM components represented in models [12–16]
Organotypic explant culture	• Preserves tissue cytoarchitecture	• Heterogeneity of explanted tissue • Imaging challenging due to low transparency [9,12]
Polarised epithelial cell culture	• Relatively high throughput	• Expensive [12,17]
Gyratory and spinner flasks	• Cheap • Easy to use • Produce a large number of spheroids	• Shear stress affects spheroid architecture [13]
Mircocarrier beads	• Cheap • Suitable for anchorage-dependent cell lines	• Require use of gyratory/spinner flasks; same problem of shear stress [12]
Artificial skin	• Able to model complex epithelial structures using support structures such as membrane inserts	• Expensive [12]
Artificial Cancer Mass	• Biomimetic • Use of plastic compression technique increases cell and matrix density	• Expensive • Difficult to culture sensitive cell lines [18,19]

models — for which the methods of production are summarized in Figure 1 — is now established as a much more accurate representation of *in vivo* conditions when compared to other *in vitro* models, such as the production of 2D monolayers [6]. This advancement is vital given the vast heterogeneity within tumors. A great number of factors must be taken into account when examining a tumor; growth, adhesion, metastasis, invasion, response to growth factors, angiogenesis, and tissue remodeling are all important considerations when producing a precise model. Cancer cells have complex interactions with surrounding cells and the extracellular matrix (ECM), and 3D tissue cultures more accurately reflect this not only on a biochemical and mechanical level but also on the level of gene and protein expression [7]. (See Figures 2 and 3).

There are two clear aims for improving cancer treatment: to improve the testing of new drugs and to increase the efficacy of drugs that are currently available. More complex tumor models more closely mimics the response seen clinically, facilitating these aims by allowing more accurate examination of drug sensitivity and resistance [8].

From a Single Cell to a Sphere

What Techniques Can Be Used to Engineer These In Vitro Tumoroids?

Conventionally, tissues are harvested *in vivo* and subsequently explanted and cultured *in vitro* [9]. Tumoroids created by xenografting retain their architecture in culture and have proven to be an effective method for many tissues, including the brain and embryonic glands [10,11]. However, these models are time consuming and expensive, and it is difficult to image these thick, light-scattering tissues without incurring photodamage [12]. Thus, culturing *in vitro* tumoroids is an alternative *ex vivo* approach that involves using isolated cells from cell lines, dissociated tissues, or stem cells [9].

3D tumoroids that accurately mimic tumor pathophysiology have been successfully engineered through numerous methods. Currently, there are seven main methods used: cellular spheroids [12–16], organotypic explant culture [9,12], polarized epithelial cell culture [12,17], gyratory [13] and spinner flasks, artificial skin [12], microcarrier beads [12], and artificial cell masses [18,19].

3D models are most commonly fabricated using cellular spheroid production techniques. This takes advantage of the natural tendency for cells to form aggregates, reestablishing cellular connections. The spheroid microenvironment enables them to differentiate, forming a tissue-like phenotype that is similar to an *in vivo* tumor [12]. Cancer models created by this technique can be formed by five main methods: rotary cell culture system [20], microarray [16], hanging drop plate technique [21,22], hanging drop array [14], or collagen-implanted spheroids [15]. These systems are capable of producing large numbers of consistently sized spheroids. Additionally, they can be easily applied to high-throughput screens using the 384 hanging drop array [14], and the spherical shape allows tumor growth and invasive processes to be modeled [12]. Apart from collagen-implanted spheroids, the cellular spheroids technique negates the need for an external scaffold [12]. Collagen-implanted spheroids are advantageous in that they contain a biodegradable scaffold.

There needs, however, to be a common consensus regarding the definition of a spheroid as it has been misused to describe loose aggregates that detach easily, lack cell–cell and/or cell–matrix interactions, or do not have a spherical geometry [16]. The complexity of these models can however be further enhanced, and there is a need for the tumor microenvironment (TM) to be better represented.

Although many methods have been developed, each method has its own set of advantages and limitations. In addition, different methods may be more suitable for studying different mechanisms. A balance needs to be struck between achievability, complexity of the tumor, suitability, and cost before deciding which method should be employed to investigate a particular mechanism.

Varying Factors to Engineer the Ideal Tumor

Tumoroid characteristics can be tailored to study various mechanisms. Factors that influence these characteristics include tumor size, which influences oxygenation and the number of cell lines used.

3D models are inherently avascular tumors and rely solely on diffusion for nutrient and oxygen transport. Hence, by altering the size of tumoroids, various stages leading up to the formation of necrotic regions can be studied. Smaller tumoroids (400 to 600 μm) would contain viable cells at the rim and core region, while larger tumoroids would instead possess a characteristic necrotic core [6]. Some studies require tissues to be thin (~0.3 mm) to enable the interior of the tissue to receive adequate oxygenation and nutrition [9]. Other studies focus on mimicking the distinctive nutrient insufficiency within the tumor's interior, which induces the

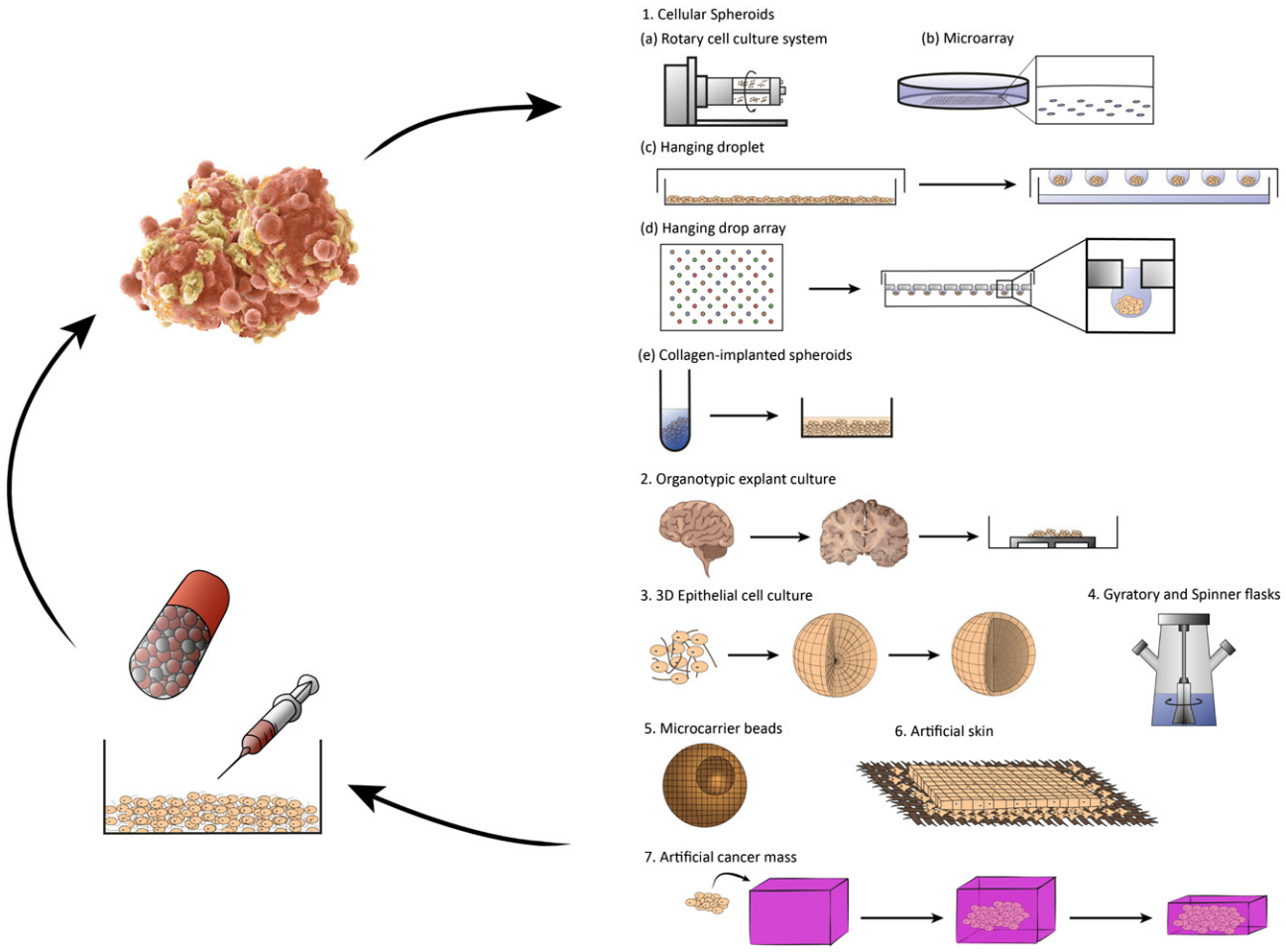

Figure 1. Summary of 3D tumor models. There are seven main methods: 1) Cancer models created by cellular spheroid technique can be formed by five main methods: rotary cell culture system [20], microarray [16], hanging drop plate technique [21,22], hanging drop array [14], or collagen-implanted spheroids [15]. 2) Organotypic explant culture involves dissecting organs into slices, which are subsequently cultured on a semiporous membrane or embedded in a collagen matrix, and grown in an air–liquid growth medium interface [12]. 3) Polarized epithelial cell culture is an approach in which cells are grown on a porous membrane, forming polarized monolayers [12]. 4) Gyratory and spinner flasks are used to culture cells in suspension; the fluid movement aids transport of both nutrients and waste, facilitating growth of the spheroid [13]. 5) Microcarrier beads made from various materials, including dextran, gelatine, glycosaminoglycans, and other porous polymers, can be used to create these tumoroids by acting as a support structure for culture of cell lines that are anchorage dependent [12]. 6) Artificial skin can also be used as a 3D culture model. It is made up of three main layers: the fibroblast and a biodegradable fibre mesh layer, which together form the dermis, and keratinocytes, which form the epidermis [12]. Fibroblasts are first cultured *in vitro* and seeded onto the fiber mesh layer. Keratinocytes are then added to the dermal tissue to form the epidermis [12]. 7) Artificial cancer masses are created by seeding cancer cells onto a collagen hydrogel. Plastic compression is then applied to enhance cell and matrix density [18,19].

formation of a necrotic core. The ability to mimic this phenomenon (nutrient insufficiency and necrosis) in a controlled setting is crucial in understanding tumor–host interactions. For instance, growing 3D models could provide better insight into mechanisms involved in angiogenesis, chemotherapy drug resistance [23], cell adhesion, and migration [12]. For instance, Hicks et al. demonstrated that the use of a 3D culture in a mouse model could predict therapeutic activity of anticancer drug tirapazamine [23]. Additionally, the oxygen and nutrient gradients would enable intratumor heterogeneity to be captured within these 3D models. Lawrenson et al. have developed a 3D *in vitro* model to study the genetic and clinical heterogeneity in ovarian epithelial cancer [24].

Aside from varying tumor size, 3D models can be bioengineered to be either simple cancer spheroids or tumoroids comprising multiple cell lines. Multicellular tumoroids are cultured from established cancer cell lines or disaggregated samples from human tumors [18].

Recently, 3D models have been able to successfully mimic the early steps of metastasis such as epithelial mesenchymal transition [25]. Studies by Fischbach et al. have shown that 3D cultured human oral epithelium cells (OSCC-3) can transition into a fibroblastic morphology and also have invasive potential [26]. More excitingly, cancer stem cells (CSCs), which are cells found within solid tumors that play a role in initiation, recurrence, and metastasis, have also been used to create 3D models. Chen et al. demonstrated the use of a nonadhesive culture system using CSCs derived from OSCC, which showed higher levels of therapeutic resistance [27]. This nonadhesive system allows spheres to detach and subsequently form small clusters, triggering anoikis due to its inability to attach [27]. Models like this

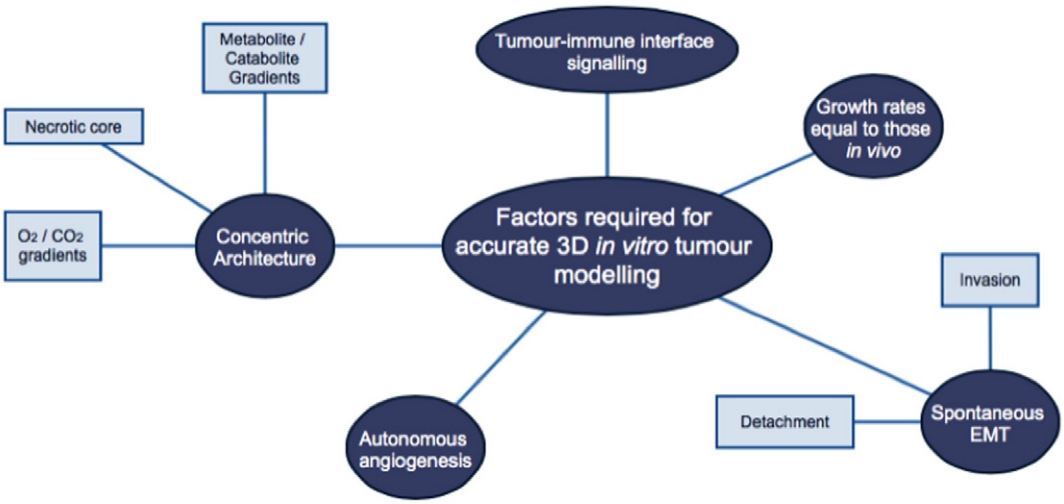

Figure 2. Figure illustrates some of the fundamental milestones which must be met before *in vitro* models can accurately recreate the tumor microenvironment.

could aid studies on metastasis and hopefully elucidate the mechanisms behind cancer cell proliferation without proper anchorage, and avoid anoikis.

The implications of such advancement could mean not only the cheaper production of more efficacious drugs and an improvement in the successful treatment of cancers but also a transition towards a system of stratified treatment. Theoretically, a tumor could be biopsied and its cells then cultured using an appropriate method, producing a 3D tumoroid. The sensitivity and resistance could then be tested to determine and subsequently produce a treatment that is tailored to the biopsied tumor. Although this would mean more effective treatment against specific tumors, it is unlikely to be implemented within the near future. Nevertheless, advances in techniques have enabled rapid (<24 hours), high-throughput generation of tumoroids [6].

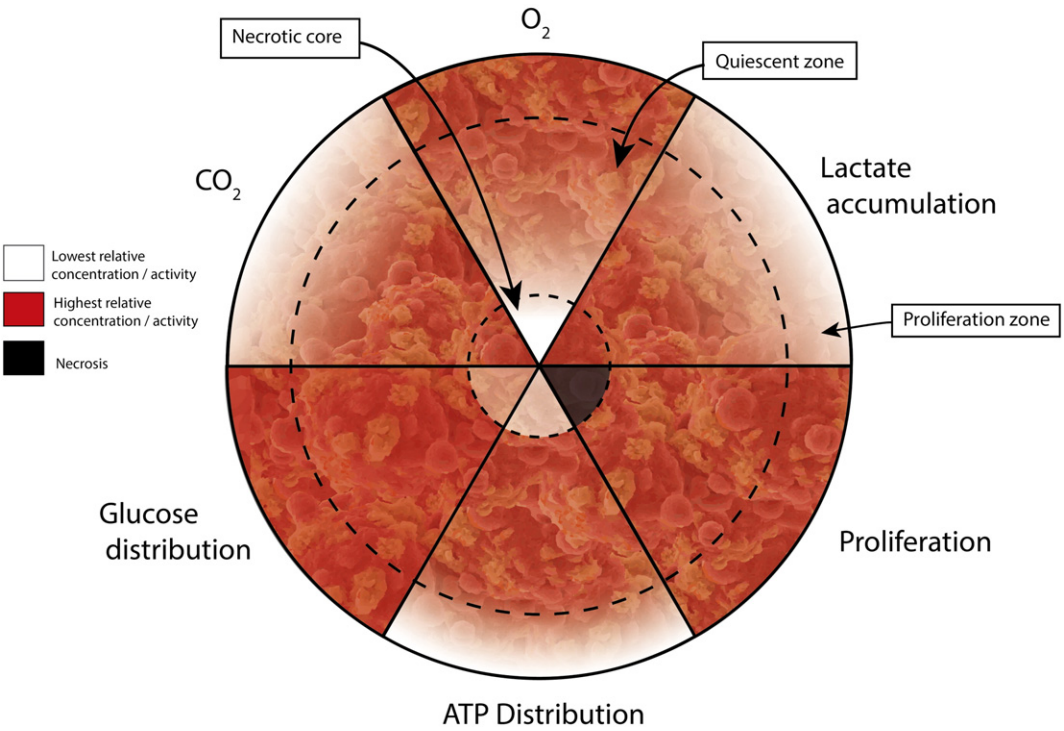

Figure 3. Illustration of the complex concentric architecture typically observed in tumoroids. Concentration gradients of nutrients and metabolites typically establish three distinct zones in the tumoroid: an outer proliferative zone, a middle quiescent zone, and an inner apoptotic/necrotic core. Proximity to vasculature ensures that there is an abundance of oxygen and glucose peripherally, along with the efficient removal of waste products, permitting high levels of cell proliferation. Centrally, low levels oxygen lead to anaerobic respiration, a buildup of toxic metabolites such as CO_2 and lactate, and subsequent cell apoptosis.

Important Considerations in Modeling the Tumor Microenvironment

In order for 3D *in vitro* models to be of use in stratified medicine, both in unlocking the specific mechanisms underlying invasion and metastasis and in allowing the assessment of drug efficacy, they must accurately recreate the complex TM. Although the TM is poorly understood, there are some fundamental aspects which are integral to the creation of any useful *in vitro* tumor model.

Concentric Architecture

Tumoroids *in vivo* display some degree of concentric architecture. Typically, tumors display three relatively distinct zones [8]: an outer mitotically active proliferative zone, a middle quiescent zone, and an inner apoptotic/necrotic zone. These somewhat distinct concentric layers are established due to the relative distance of cancer cells from the surrounding vasculature. The outermost cells, being closest to the vasculature, benefit from rapid removal of toxic metabolites and ready access to nutrients and oxygen [16]. For instance, Nyga et al. demonstrated the presence of concentricity through the measurement of the oxygen gradient through a tumoroid using a real-time oxygen probe [18]. In the presence of oncogenic mutations and the vast supply of nutrients, they are free to rapidly proliferate with low concurrent levels of apoptosis. In contrast, the innermost cells of a tumor undergo apoptosis and necrosis due to chronic pathological hypoxia and the accumulation of toxic metabolites.

An accurate 3D *in vitro* tumor model must ensure that this architecture is established and maintained since varying pH levels throughout the tumoroid greatly affect drug distribution. Raised levels of lactate in the necrotic core decreases pH centrally, and hence the actions of weakly basic drugs, such as chlorambucil and mitomycin C, are potentiated. In contrast, weakly acidic drugs such as mitoxantrone and anthracyclin will often show good efficacy in monolayers models, but their poor distribution to the acidic core in 3D means that their overall action is often seen to be attenuated *in vivo* [8].

Detachment & Invasion of Surrounding Tissue

Tumors *in vivo* will typically undergo cell–cell and cell–matrix detachment and extravasation and form metastases. This process is essential in any invasive tumor, and cancer models should replicate this [28].

A recent study by Nyga et al. has successfully demonstrated the successful detachment of HT29 colorectal cancer cells seeded in a type I collagen matrix populated with fibroblasts and epithelial cells [19]. The mechanisms behind this detachment were not elucidated in the study; however, other literature suggests that integrin class-switching may be at least partially responsible for cell detachment. Hanahan et al. propose that integrin isotypes $\alpha_3\beta_1$ and $\alpha V\beta_3$ may be particularly useful in promoting the detachment of cells from their native ECM in favor of ECM that is highly protease susceptible [29], hence encouraging rapid metastasis. Specifically, $\alpha V\beta_3$ may play an important role preventing anoikis when metastatic cells encounter *ex situ* ECM due to its relatively unspecific ligand binding [30]. Future research efforts should concentrate on analyzing the metastasis of tumoroids from a native ECM TM to a protease-susceptible, prometastatic form; 3D *in vitro* models should explicitly assess the mechanism by which metastatic cells integrin class-switch to cause extravasation to lymphatic tissue, although involvement of the PI-3 K pathway is strongly suspected [31].

Cell Microenvironment

ECM stiffness has been found to regulate gene and protein expression in cells, affecting cell morphology and behavior via integrin mediated cell–cell signaling, which has an established role in cancer progression [25]. Dynamic forces such as flow, stretch, and diffusion are important components of the cell microenvironment [32]. For example, it is known that shear forces exerted by fluid flow affect angiogenesis and the laying down of endothelium, which stabilizes the vasculature [33]. As such, there has been increasing interest in the effects of a dynamic and mechanical microenvironment on cancer cells and its surrounding stroma [32]. In 3D models, mechanical stresses can be altered through the selection and/or modification of the scaffolds, allowing further research into this phenomenon [26,33]. ECM stiffness modeling is important in allowing us to understand and mimic the malignancy of the tumor and thus further aids drug development.

Immune Interface

The concept of a reactive stroma is that immune responses synergize with other processes to cause a characteristic stroma that promotes cancer progression [9,25]. For example, an increased efficiency in the induction of endothelial cell tubule formation was seen *in vitro* with the co-culture of cancer cells with macrophages. This was believed to be caused by the release of inflammatory cytokines by the macrophages [34].

Furthermore, there is evidence to suggest that the cells of the hypoxic core may be protected from routine immune surveillance *in vivo* due to high local concentrations of lactate substantially downregulating the effects of cytotoxic T-lymphocytes [35]. Therefore until more accurate tumor-immune interfaces are created, the complex regulatory feedback systems will remain unaccounted for, limiting the extent to which the models can mimic the *in vivo* scenario.

Co-Culture and Its Significance on the TM

Co-culture of multiple cell types is essential in accurately creating the TM. Stromal cells not only influence tumor cell behavior through paracrine signaling but may also change cell behavior by altering the mechanical properties of the support scaffold. One of the major types of stromal cells is carcinoma-associated fibroblasts. Carcinoma-associated fibroblasts secrete cytokines, growth factors, and also matrix metalloproteases (MMPs) to remodel the ECM [36]. Pinilla et al. demonstrated that co-culture of breast cancer cells with human adipose-derived stem cells can cause upregulation of MMP-9 due to the presence chemokine ligand 5 [37]. This is significant because upregulation of MMP-9 not only is key to the process of desmoplasia but also may act as a key angiogenic "switch" [38].

Stromal cells regulate interstitial fluid volume and therefore interstitial fluid pressure. This is particularly pertinent in tumoroid modeling, as increased interstitial pressure has been associated with greater levels of drug resistance and cell survival [39–41]. Thus, models that do not incorporate stromal cells may be prone to giving inaccurate drug efficacy results.

It is established that co-culturing, which refers to the incorporation of multiple cell types, can more accurately replicate intratumor heterogeneity [29]. However, more research is needed in both stromal and immune cells before *in vitro* models successfully replicate the *in vivo* TM.

How Do 3D Models Improve Pharmaceutical Testing?

Cancer is not a single disease, and it does not remain static in a given patient. As treatment prognosis varies depending on location and

timing, personalized therapy remains the ultimate goal of cancer treatment. At present, empirical therapy, which yields poor responses, is still utilized. In non–small cell lung carcinoma, third-generation drugs only achieve a 40% response rate [42]. With the maturity of 3D tumor modeling techniques, tumoroids can be used to replicate both primary tumors and metastases for chemosensitivity tests to determine the best course of treatment for the individual patient and hopefully increase response rates.

Evidence for the Use of 3D Models

Previous studies have used collagen gel droplet embedded culture drug sensitivity testing (CD-DST) as a simplistic 3D model to determine chemosensitivity in patients. CD-DST is a technique where a suspension of tumor cells is added to a type I collagen solution and the mixture is allowed to gel [42,43]. In one study, patients treated with *in vitro* sensitive drugs showed a response rate of 85.7%, whereas patients treated with *in vitro* nonsensitive drugs only showed a response rate of 41.7%. Evidence from other trials also shows that drugs proven to be effective *in vitro* show better clinical results *in vivo* [42–45]. Despite the lack of modification to the TM, CD-DST still shows extremely promising results. It follows that the increased system tunability afforded by the advances in tumor modeling techniques would greatly enhance the translatability of *in vitro* drug testing results to *in vivo* drug response.

Angiogenesis and Drug Response

Correa de Sampaio et al. recently developed a "Minitumor Spheroid" 3D model incorporating endothelial cells, fibroblasts, and cancer cells. When grown in a collagenous scaffold, capillary-like sprouting was observed. A network of capillary structures with lumens was observed after prolonged incubation. Similar to the *in vivo* scenario, the vasculature responded to treatment with bevacizumab, an anti-VEGF antibody, showing inhibition of angiogenesis [34].

Antiangiogenic agents thalidomide and endostatin demonstrated successful inhibition of tumor angiogenesis in murine and crude *in vitro* models. However, both drugs failed to show a significant effect clinically. In concordance with clinical data, both drugs showed no effect in the Minitumor. The success of the Minitumor in being an accurate predictor of clinically efficacious drugs makes this a vast improvement over past models, which mostly failed to incorporate cancer cells, resulting in overly simplistic, unrepresentative models [34].

The study of this model may also shed some light on tumor-associated vasculature, which is unlike vessels in healthy tissues. It is often tortuous and leaky, resulting in poor perfusion and drug delivery [46]. The interstitial fluid pressure of the tumor is often raised and is believed to compress the vasculature, reducing perfusion [36,47]. Together, these make drug penetrance very difficult, and this is an important determinant of efficacy [48]. Understanding the mechanisms of tumor-associated vasculature may then put us in a better position to design drugs that can reach its target. Nevertheless, pharmaceutical testing in 3D models is still in its infancy, as techniques for developing these models have yet to be standardized and optimized.

Cancer Stem Cells

Recent studies have discovered a subset of tumor cells known as CSCs. They possess characteristics similar to stem cells, including the ability to self-renew. CSCs are believed to be resistant to chemotherapy, persisting after treatment and potentially causing cancer recurrence [27,49–51].

In 2008, Li et al. found that there was a statistically significant increase in the proportion of CSCs in patients postchemotherapy. However, there was no significant change in CSC proportion in patients who received lapatinib treatment followed by chemotherapy. The CSC population, being less sensitive to chemotherapy, increased in proportion without the adjuvant use of lapatinib. Lapatinib is an epidermal growth factor receptor and HER2 inhibitor. It is believed that epidermal growth factor receptor signaling is essential for CSC self-renewal, and this explains why better control of CSCs was achieved in the lapatinib group [52].

The work of Li et al. clearly demonstrates the insufficiency of conventional chemotherapy. It is currently unclear exactly how self-renewal of CSCs is regulated and their role in metastasis. More studies must be done on CSCs to elucidate their characteristics. 3D cultures allow that to happen and are also an avenue to test CSC-targeted therapies [52].

Models grown from CSCs derived from primary tumors have shown to mimic the *in vivo* behavior of the cancer cells more accurately than those grown from established cell lines [50,53,54]. One challenge is obtaining CSCs from a biopsy, as these cells are a subpopulation of the tumor. Therefore, techniques to identify CSCs should be incorporated into our proposed patient pathway. By identifying and obtaining CSCs, we are then able to expand them *in vitro* and thus circumvent the inaccuracies that stem from the use of established cell lines.

Challenges, Future Direction, and Conclusion

The patient pathway that we have proposed is an ideal that we strive towards. However, there are some limitations. A biopsy of a tumor only obtains a small proportion of the cells. The heterogeneity of the tumor cell population means that the tumor model cultured may not be entirely representative of the primary tumor. As previously mentioned, another limitation is that CSCs may not be present in the biopsy sample. Although not fool-proof, multiple biopsies can be taken from different parts of the tumor to try to avoid this problem. It may also be possible that, in the future, CSCs could be labeled, facilitating their extraction during the biopsy.

For 3D modeling to be universally adopted, it must be cheap and efficient, providing rapid results that can be translated to the clinical situations. For this to happen, the process of culturing the tumor must first be streamlined and standardized. Commercially available culture kits would be the ideal solution. A high-throughput method of analyzing the chemosensitivity of the tumor must then be devised and implemented. This would allow for convenient, consistent culturing of the models and efficient analysis, culminating in rapid results that can be translated into feasible treatment options for the patient.

At the same time, much more work needs to be carried out, especially in the areas of simulating metastases, angiogenesis, and the immune interface. In each of these areas, there have only been reports of a few isolated successes. We are probably a distance away from fully emulating the *in vivo* scenario in these areas. Nevertheless, current tumor models have yielded very encouraging results, and with the vast amount of research that is being carried out, personalized *in vitro* modeling is no longer a fantasy.

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