Current Topics in Peptide & Protein Research
Vol. 18, 2017

Review

Novel use of peptides to facilitate the formation of 3D multicellular tumor spheroids

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ABSTRACT

To test the efficacy of novel antitumor drugs and delivery systems, *in vitro* models that mimic solid tumors are necessary. Three-dimensional (3D) models such as multicellular tumor spheroids (MTS) have been deemed superior to two-dimensional (2D) cell cultures due to their ability to mimic the 3D nature of solid tumors. Although several methods exist for spheroid generation, they fail to mimic many of the intricate *in vivo* interactions between cancer cells as well as between cancer cells and the extracellular matrix (ECM). The issues are that these approaches make it difficult to ascertain the efficacy of drug therapies. Here, we review the importance of 3D models and the components of the tumor microenvironment (TME) and ECM that are required to recapitulate the complex interactions in clinically relevant *in vitro* models. We also discuss classical spheroid models as well as novel methods that attempt to recapitulate the TME to a greater extent. We focus on the use of the cyclo-RGDfK peptide and its modification with triphenyl phosphonium cation (TPP), namely cyclo-RGDfK(TPP). Within the ECM, the RGD (Arg-Gly-Asp) motif in fibronectin has been found to promote cell-to-cell and cell-to-matrix interactions. The chemically engineered cyclo-RGDfK(TPP) peptide is capable of strategically mimicking the ECM within the TME to facilitate 3D MTS formation from 2D monolayer cancer cells. Additionally, this peptide allows for a reproducible method that directly allows for the formation of tighter spheroids that can be theoretically applied to co-culturing experiments as an efficient *in vitro* model to study the effects of antitumor therapies.

KEYWORDS: multicellular tumor spheroids, cyclo-RGDfK, peptides, extracellular matrix, tumor microenvironment, mimicry.

ABBREVIATIONS

CAF	:	Cancer-associated fibroblast
CSC	:	Cancer stem cells
CyclicRGDfK	:	Cyclic Arg-Gly-Asp-D-Phe-Lys
EPR	:	Enhanced permeation and retention
ECM	:	Extracellular matrix
LAK	:	Lymphokine-activated killer
LOX	:	Lysyl oxidase
MCS	:	Multicellular spheroids
MMP	:	Matrix metalloproteinase
MTS	:	Multicellular tumor spheroids
PEI	:	Polyethyleneimine
TME	:	Tumor microenvironment
TPP	:	Triphenyl phosphonium

INTRODUCTION

The efficacy of antitumor drug discovery and delivery is affected by several elements within the tumor microenvironment (TME). As such, understanding the TME in the context of the intricate interactions

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between stromal cells such as fibroblasts and immune cells within the extracellular matrix (ECM), as well as the blood vessels that supply oxygen and nutrients, is critical to the development of successful therapies [1]. Experimental drug development and delivery have relied on two-dimensional (2D) *in vitro* cancer cell monolayer models before moving forward to preclinical animal models which have been increasingly scrutinized for both ethical and cost-related concerns [2]. A significant and growing area of cancer research is developing three dimensional (3D) modeling systems that act as a viable intermediate between *in vitro* and *in vivo* studies. Not only do these modeling systems need to be physiologically accurate but they must also be reproducible and cost-effective in order to advance cancer drug development and delivery. 3D multicellular spheroids (MCS) are a unique disease modeling system that can mimic and investigate micro-environmental factors that impact tumor therapy [3]. However, a challenge in the field that remains surrounds currently available 3D models being incapable of efficiently recapitulating the complexity of the TME, including the ECM and complex cellular interactions [3]. Interestingly, chemically engineered peptides and polymers have provided a unique conduit for facilitating the formation of MCS from 2D monolayer cultures that can simulate the cellular and biochemical complexity of the tumor and its surrounding microenvironment, namely the ECM and TME.

The relevance of modeling the tumor microenvironment

The importance of an intermediate 3D modeling system is to mimic the interactions seen *in vivo* to efficiently study the efficacy of multiple treatment options as well as their interactions *in vitro* [2]. Establishing useful 3D models may relieve the need for preclinical animal studies before advancing to human clinical trials due to their potential to mimic a spectrum of autocrine, paracrine and cell-specific behaviors seen *in vivo* which cannot be captured in 2D systems [3].

The tumor microenvironment plays a crucial role in malignant progression and metastasis, significantly influencing drug delivery and therapeutic efficacy [4]. The TME is characterized by leaky endothelial cells within the vasculature, poor oxygenation resulting in a hypoxic core, a low pH, high interstitial pressure, and the enhanced permeation and retention effect (EPR) which has been previously used to target the solid tumor. Thus, reproducing the EPR effect *in vitro* is an essential factor to consider when attempting to establish the most clinically relevant 3D model [5]. Within the TME, a significant component is the highly fibrillary extracellular matrix that is present in the interstitial space [6]. The ECM is heterogeneous and dynamic, composed of proteins, glycoproteins, proteoglycans, and polysaccharides with different physical and biochemical properties [7, 8]. The components of the ECM are subject to remodeling by matrix metalloproteinases (MMPs) and lysyl oxidases (LOX) in response to biochemical stimuli within the TME, ultimately facilitating several processes implicated in tumorigenesis [7, 8].

An abnormal ECM is a hallmark of cancer progression and has been shown to promote the formation of a TME that influences the behavior of stromal cells, including endothelial cells, immune cells, and fibroblasts, all of which are known to promote abnormal ECM production [7]. Thus, the abnormal ECM exacerbates the local specialized niche in a feedforward manner and continues to promote a highly tumorigenic microenvironment. Cancer-associated fibroblasts (CAFs) in the TME have been demonstrated to play a significant role in cancer progression and drug resistance, whereby the overexpression of LOX *in vivo* results in the stimulation of collagen cross-linking and stiffness that promotes cancer cell invasion, contributing to malignant growth and metastasis [9, 10]. These fibroblasts are a significant source of fibronectin, an extracellular glycoprotein implicated in cell adhesion, migration, growth, and differentiation [11]. Fibronectin primarily mediates its function through integrins, αβ heterodimeric cell membrane receptors that have bidirectional signaling, and acts as a link between the ECM and the intracellular cytoskeleton [12]. Fibronectin contains multiple integrin-binding sites, although the most studied is the RGD (Arg-Gly-Asp) sequence [13]. Integrin activation is accompanied by conformational changes in the 3D structure of the integrin that facilitates adhesion to ECM ligands and links the extracellular contacts to the cellular cytoskeleton [12]. As a result, the incorporation of CAFs into *in vitro* 3D models will result in a more clinically relevant tumor model due to their essential role in cancer invasion.

Multicellular tumor spheroids as a modeling system

3D multicellular tumor spheroids (MTS) present a more physiologically relevant platform for the delivery and testing of chemotherapeutics for several reasons. Firstly, they can simulate the phenotypic heterogeneity of the tumors which results in altering the proliferation rate, gene expression, and differentiation, giving rise to morphological and functional changes [14]. This MTS platform makes targeting the tumor by a chemotherapeutic agent more challenging. The clonal selection theory explains this phenomenon by assuming that this heterogeneity is created by a genetic instability secondary to the nutrient deprivation that leads to cell mutations [15]. Since 3D spheroids have oxygen and nutrient gradients, they could demonstrate heterogeneity with increasing similarity to the TME.

Secondly, similarly to *in vivo* environments, growing cells in the form of 3D spheroids can increase resistance to chemotherapies. Cells in the core of spheroids are protected from therapy by the cells on the outer layer [16]. The ability of 3D models to simulate this sequestration phenomenon that is seen in cancer cells *in vivo* has helped researchers better understand the mechanism of drug resistance wherein these inner cells recapitulate the necrotic core of a tumor. 3D spheroid models have allowed for a thorough investigation of these mechanisms as well as testing of multidrug therapy regimens *in vitro* prior to proceeding to preclinical animal models and ultimately human clinical trials.

Cancer stem cells (CSCs), which are responsible for the relapse of cancers after treatment, have been cultured successfully as spheroids. These spheroid cultures maintain critical properties of stem cells, including gene expression profiles, colony-forming and tumorigenic activity, differentiation potential, cytokine secretion, and resistance to chemotherapy [17]. Moreover, studies show that some cells in the spheroid model exhibit CSC-like characteristics, including slow proliferation rate, self-renewal and an undifferentiated phenotype that can undergo multilineage differentiation [18].

Cells grown in 3D cultures generate different gene expression patterns similar to that seen in human tumor cells [19]. Gene expression is a result of the interaction with the ECM and stromal cells, with gene expression changes driving changes in morphology, proliferation rates, and drug resistance, all of which have been demonstrated in 3D models and significantly mimic the *in vivo* cancerous tissue [20]. 3D models allow for the mimicry of intercellular signaling between different cell types *in vivo*. Applying co-culturing techniques to spheroid formation can help decipher how multiple cell types found in tissues *in vivo* might impact drug delivery [17].

Classical 3D models and their limitations

The development of accurate 3D models that attempt to reach clinical relevance often come at the expense of efficiency, reproducibility, and cost [21]. Although the currently utilized 3D models allow for a means to study the pharmacokinetics and pharmacodynamics of drug delivery, chemoresistance, and tumor growth, several challenges remain.

Several of the classical methods of generating MCS involve the use of mechanical or gravitational forces. In brief, the agarose method requires the use of an agarose scaffold with uniformly sized micro-wells in cell culture plates for cell seeding, allowing for a reproducible mass production of the MTS model [22]. The rotary method produces MTS that vary based on oscillation rates and cell concentrations, with the rotator base of the cell culture permitting dual oxygenation of the medium from both superior and inferior exposure, which in combination with high oscillations at 15 Hz prevents hypoxia [23]. The hanging drop method exploits gravity to allow cells to aggregate at the base of the droplet and adhere to each other due to contact, resulting in a reproducible model for cells that were previously not responsive to the rotary method [24, 25]. Lastly, the scaffold method involves the use of a matrix for cells to be seeded, followed by subsequent polymerization such that cells are incorporated into 3D porous scaffolds, mimicking the resistance and expression of angiogenic factors seen *in vivo* [26].

Each of the methods above is limited by various factors, as outlined in Table 1; however, as it relates to drug development, the most critical drawback

Table 1. Classical methods of 3D cancer models and their associated drawbacks.

Model	Drawbacks/Limitations
Agarose gel method	Formation of MTS is difficult [17, 21, 27-29] Limited by mass transference and cell viability [17, 21, 27-29]
Rotary method	Use of special equipment [30, 31] Lack of an individual compartment for each sample [30, 31] Absence of uniformity control (i.e., spheroid composition and size varies [29, 32])
Hanging drop method	Labor-intensive and large-scale production is difficult [33-36] Not efficient, low throughput and long-term culture is difficult [32, 34-38] Tedious handling, time-consuming, and not stable [32, 34-38] Although advances in high throughput production have been made, tracking these spheroids during formation is difficult [39]
Scaffold technology	Sample retrieval for further analysis is complicated and scaffold material biocompatibility and biodegradability is problematic [40-43] Expensive and requires specialized equipment for scaffold fabrication and involves pooled screening which is limited by low yield [21, 28, 29, 44]

is that the cellular and ECM interactions observed *in vivo* are absent. As outlined above, mimicking these interactions when modeling tumor progression and developing drugs is essential to arrive at accurate conclusions regarding drug efficacy.

Novel methods for generating spheroids mimicking the complexity of tumors

Designing novel 3D culture systems has become an area of intense research interest. There are several new modeling systems and peptides that are being considered to facilitate the 3D cancer cell interactions observed *in vivo*. One model system is seen in organoids which are 3D structures generated from a population of adult stem cells harvested from an organ of interest and are allowed to organize into 3D spheres with epithelial architecture resembling the organ of origin [45]. Theoretically, once isolated, CSCs could be used in the same method that is used to generate non-malignant organoids. However, a drawback remains in that the complexity of the additional cells involved in the establishment of a tumor would not be present in a cancer organoid since they would be of non-malignant origin.

Therefore, an additional aspect of 3D culture systems is not only mimicking the TME or the ECM, but also the interactions between the phenotypically diverse cells that can be found in the tumor. However, very few modeling systems have been able to successfully overcome this unique challenge. As such, co-culturing methods are required to introduce this complexity to 3D modeling systems. Recently, Lao *et al.* [46] reported on a novel culturing system that can mirror these interactions and cellular diversity to generate high-throughput testing on novel treatment options. Their method involves adding a droplet of cells at a high density and low volume to the center of an agarose-coated concave surface to create a 3D spheroid consisting of a mix of 25% fibroblasts and 75% tumor cells. Once generated, these cells were viable for two to three weeks. Although this method was reproducible, the number of spheroids generated (i.e., one per well) introduces an added challenge wherein the drug efficacy would need to be tested on multiple wells containing single isolated spheroids, making this method very labor-intensive and unfeasible. Furthermore, due to the nature by which the spheroids were generated, the change in media could potentially disrupt the cellular interactions, as the 3D conformation is gravitational and mechanical. Another co-culturing study by Hsiao *et al.* [47] involved a similar process in which cells were mixed in various ratios and spheroids were then generated using microfluidic systems. In brief, their method involved a two-layer microfluidic system consisting of prostate cancer cells, endothelial cells and osteoblasts, and ensured that each spheroid incorporated an equal quantity of these cells. This particular technology proved to be useful in establishing a niche that was able to recapitulate the complex interactions between various cell types. However, one crucial caveat from

this study was the importance of understanding which cells should be co-cultured together to ensure that cross-talk between cells does not lead to a lack of proliferation or cell death, but instead is capable of maintaining growth and cell viability.

While the methods above were able to mimic the complex cellular interactions seen *in vivo*, the fact remains that the methods used required precision and the use of multiple instruments, leaving room for error. Thus, a more feasible and practical method to quickly generate a spheroid is required. Ong *et al*. [48] reported on a scaffold-free method designed with an inter-cellular linker that was capable of facilitating cellular adhesion. The linker was generated using polyethyleneimine (PEI) on a positively charged backbone. Although the linker had a half-life of two days, the spheroids were generated in seven days, compared to the previous time of 16 days required by the rotary method. The method described applies to any cell type as it is non-specific. Unfortunately, this method was not applied to co-culturing methods.

Synthetic peptides for 3D spheroid formation – the cyclo-RGDFK(TPP) method

Although the novel methods described above show promise, a far more straightforward and efficient method that is capable of circumventing many of the limitations outlined above is required to assess drug efficacy rapidly. Akasov *et al*. have recently described a novel one-step, reproducible method of spheroid formation using cyclic (Arg-Gly-Asp-D-Phe-Lys) RGDfK peptide (cyclo-RGDfK) and cyclo-RGDfK conjugated with triphenyl phosphonium cation (TPP) known hereafter as cyclo-RGDfK(TPP) [49].

The rationale for using the cyclo-RGDfK(TPP) peptide

As previously discussed, a significant challenge in drug development and delivery is understanding the contribution of the various components within the TME to the overall process of cancer development. The ECM, in particular, the production of fibronectin by CAFs facilitates cell-cell adhesion and cell-ECM adhesion through integrins [12]. The RGD peptide domain within fibronectin is the attachment site of not only the family of integrins with an RGD-binding motif but also of several other adhesive proteins [50]. Recognition of this RGD peptide sequence by integrins has been associated with signaling pathways that modulate cytoskeleton organization, cell growth, motility, and channel activation [51]. This peptide sequence, although small, can be significantly altered by various amino acid substitutions, and is therefore very specific in its activity. Even substitutions such as replacement of aspartic acid with glutamic acid, or glycine with alanine, cause a 100-fold reduction in the peptide's activity in cell attachment assays. Moreover, the conformation of the residues is critical for peptide activity. While the D-configuration of aspartic acid results in an inactive peptide, RGD peptides with D-arginine are active [50].

As the recognition of the RGD motif by $\alpha_5\beta_1$ integrins facilitates cell-cell and cell-matrix, it was postulated that synthetic RGD peptides should mimic the natural peptide and its interactions with integrins [49]. Following testing of five RGD compounds, two of which were cyclic and three of which were linear, on 12 different cell lines, it was found that the linear RGD peptides did not reveal any physiological effects, whereas the cyclic peptides resulted in spheroid formation [49]. These findings suggest that synthetic cyclic RGD peptides can mimic the natural proteins found within the ECM to facilitate a 3D tumor model. These results are consistent with current literature that reports that linear RGD peptides inhibit spheroid formation, but natural ECM proteins favor spheroid formation [52]. Of the two cyclic peptides, one was cyclo-RGDfK, containing the RGD domain of fibronectin within the ECM, and the other was conjugated with the TPP cation. Amongst these two cyclic peptides, MTS formation using cyclo-RGDfK(TPP) was consistently more efficient than cyclo-RGDfK, resulting in a lower peptide concentration required for spheroid formation; however, both peptides resulted in no cytotoxic effects [49]. The rationale for utilizing cyclo-RGDfK peptides is due to their action as selective inhibitors for the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. These subgroups are of particular interest concerning cancer research due to their implication in tumor-induced angiogenesis [53]. The covalent attachment of the TPP cation, though commonly studied as a mitochondrial antioxidant [54], to the cyclic RGD peptide was incorporated due to the enhanced electrostatic interactions that would facilitate cell self-assembly [55].

Process of MTS formation

An advantage of this biochemical method of spheroid formation using the cyclo-RGDfK(TPP) peptide is the simple one-step experimental process as demonstrated in Figure 1. A pre-determined amount of the peptide is added to cultured 2D cell monolayers, and without the need for mechanical or gravitational forces, a spheroid is formed within seven days of peptide addition [49]. Although the experimental set-up is one-step, the outcome is a result of three steps that take place at the cellular and molecule level as depicted in Figure 2. The addition of the peptide results in the formation of loose cellular aggregates due to cyclo-RGDfK(TPP) peptide binding with $\alpha_5\beta_1$ integrins on the cell surface [56]. This approach is followed by a "latent" period in terms of the compaction of the formed aggregates due to the accumulation of cell-surface E-cadherin expression [33]. Once there is an accumulation of E-cadherin, tight spheroids are formed as a result of increased E-cadherin-E-cadherin interactions between cells [56]. Due to integrins being highly glycosylated receptors [57], it was thought that glycosylation played a role in this cell-cell adhesion. Indeed Akasov *et al.* demonstrated that sialyation transforms malignant cells into 3D MTS using the cyclic-RGDfK(TPP) method [58]. Previous studies have reported that the removal of sialic acid from the surface of mammary cancer cells eliminated cell-cell adhesion *via* E-cadherin as demonstrated by aggregation assays [59]. To confirm the importance of sialic acid residues in spheroid formation, neuraminidase, an enzyme that has been reported to prevent cell adhesion and aggregation and cleaves sialic acid residues [59], was used to treat mammary cells in the presence of cyclo-RGDfK(TPP). After significantly reduced spheroid volumes were observed compared to spheroids without neuraminidase treatment, these results were confirmed with lectin inhibition for α2,3-sialic and acid, α2,6-sialic acid [58].

The importance of sialylation in the formation of MTS in pancreatic and breast cancer cells was confirmed in the formation of prostaspheres using cyclo-RGDfK(TPP) by Haq *et al.* [56]. They demonstrated that relative levels of α2,3-sialic

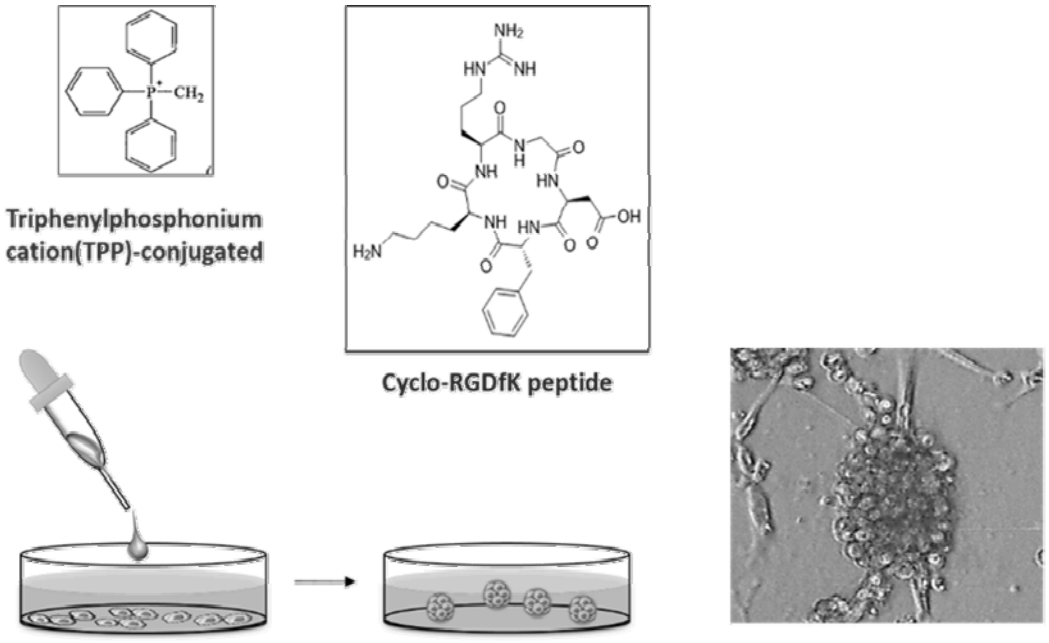

Figure 1. Experimental process for MTS formation using the cyclo-RGDfK(TPP) method. The top two images depict the chemical structure of the peptide used to generate spheroids. The bottom images depict the simple method by which spheroids are produced. In brief, a previously established concentration of the peptide is added to cells in monolayers. After seven days, 3D spheroids are generated without the addition of any mechanical or gravitational force or changes in the components. Once formed, additional assays and tests can be conducted.

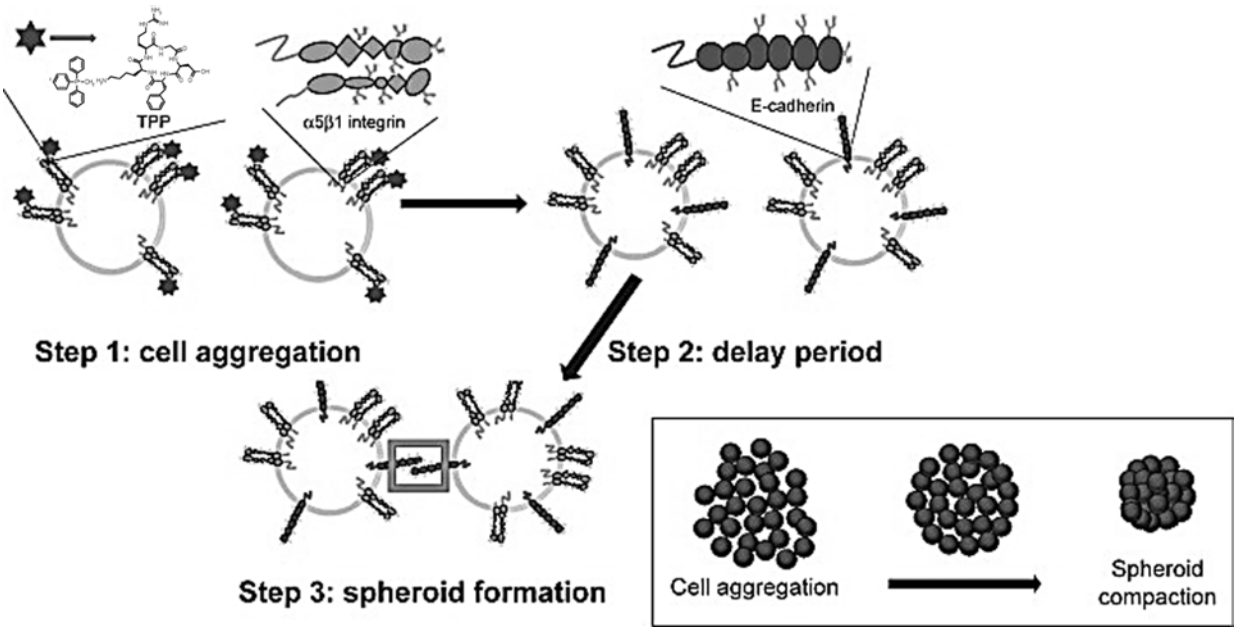

Figure 2. Cellular steps in the formation of 3D MTC spheroids. Addition of cyclo-RGDfK (TPP) to 2D monolayers results in the interaction of the peptide with α5β1 integrins on the surface of cells leading to cell aggregation. This approach stimulates E-cadherin expression and ultimately results in spheroid compaction. Taken in part from: Haq, S., Samuel, V., Haxho, F., Akasov, R., Leko, M., Burov, S. V., Markvicheva, E. and Szewczuk, M. R. 2017, OncoTargets and Therapy, 4(10), 2427. Publisher and licensee Dove Medical Press Ltd. This is an Open Access article which permits unrestricted non-commercial use, provided the original work be properly cited.

acid and α2,6-sialic acid are correlated with the ability of prostate cancer cells to form prostaspheres. This finding is particularly relevant in cancer drug development and delivery since glycosylation has been demonstrated to be implicated in the hallmarks of cancer, such as the development of resistance [60]. With this biochemical method, it was interesting to note that the required peptide concentration was found to be a function of malignancy, such that increasingly malignant cell types required less peptide to generate spheroids [49]. These results suggest that there is some correlation between metastatic potential and spheroid formation.

This one-step reproducible method of generating MTS is superior to previous methods as it does not rely on the addition of any mechanical or gravitational forces, is not cytotoxic, and the spheroids are not necessarily stuck on a surface and can be floating, or matrix-free [56]. In this way, the formed spheroids recapitulate several factors of tumors seen *in vivo*, including the essential fibronectin-integrin interactions and the facilitation of cell-cell and cell-ECM adhesion via E-cadherin. However, the inability to form spheroids in all 12 studied cell lines may be due to the difference in the expression level of integrins with RGD-binding motifs within the cell membrane [49]. This may be a limitation of this biochemical method, and additional research would be necessary to confirm the affinity of this peptide to additional integrins. Although this reproducible one-step method mimics the fibronectin component of the ECM in facilitating cell adhesion leading to the spheroid formation, it fails to incorporate additional cell types such as fibroblasts and endothelial cells that would be implicated in angiogenesis *in vivo* [61]. As such, future applications of the cyclic-RGDfK (TPP) method of spheroid formation should incorporate co-culturing of additional cell types alongside malignant cells to enhance similarity to the TME seen *in vivo*.

Limitation and future directions in the field

Although the advantages of 3D spheroids are widely recognized, they have some limitations when compared to the *in vivo* models. Firstly, with the

increase of cell complexity and tortuosity within the 3D models, it is becoming more difficult to extract all cells for analysis [62]. There is a limited degree of differentiation and availability of appropriate cells for mixed cultures as compared to the *in vivo* models [62]. Several investigators have used spheroids for studying the interaction of cancerous tissue with innate defense cells and with lymphokine-activated killer (LAK) cells. These studies showed the resistance of malignant cells to treatment with LAK cells or recombinant interleukin-2. In the 3D spheroids, the penetration of LAK cells into multicellular spheroids was relatively weak. The authors were able to demonstrate a substantial difference in both penetration and toxicity between LAK and peripheral blood cells [63]. There is an inherent difficulty to optimize 3D cell culture assays for dose-dependent cell viability, cell-cell/cell-matrix interactions, and cell migration in order to determine the cancer cell response to drug interaction, in addition to temperature and pH conditions when compared to the *in vivo* models [62].

Additional glycoproteins on the surface of cancer cells that could be used for the generation of novel 3D modeling systems are fucose residues. Fucosylation, or the modification of glycans with fucose residues, occurs ubiquitously in the human body. However, similarly to aberrant sialyation, altered fucosylation is a characteristic alteration in tumorigenesis that has been implicated in malignant transformation, invasion, and metastasis in many cancer types [64]. Recent studies have indicated that highly metastatic cancer cells expressed increased levels of core fucosylated E-cadherin [65], a variant of the critical cell-adhesion molecule, E-cadherin. Upon fucosylation, E-cadherin exhibits loss of function due to the asymmetric conformation of N-glycans on core fucosylated E-cadherin [65]. Therefore, additional studies could theoretically be done to create novel modeling systems that exploit the interaction between core fucosylated E-cadherin to generate spheroids. For example, fucosylation inhibitors may be useful in restoring the critical function of E-cadherin in cell-cell adhesion in highly metastatic cancer cells, which may contribute to the formation of superior spheroids. Characterizing fucosylated proteins in MTS is not only instrumental in deciphering the complex interplay between fucosylation and cancer progression but may also be useful for enhancing the available 3D spheroid models with specific peptides.

CONCLUSION

Although there are several limitations in the use of MTS models, their increasing complexity has resulted in the creation of a reliable and cost-effective tool as an adjunct to cancer research. Through further development of 3D tumor spheroids, MTS models may provide enough reliable information regarding cancer biology and chemotherapeutics that the need for animal and human testing will decrease drastically.

ACKNOWLEDGEMENTS

This work was supported in part by grants to M. R. Szewczuk from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Encyt Technologies, Inc.

M. Sambi is a recipient of the Queen's Graduate Award (QGA). B Qorri is a recipient of the QGA and the 2017 Terry Fox Research Institute Transdisciplinary Training Program in Cancer Research. R. Kalaydina is a recipient of the QGA.

CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest in this work.

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