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Three-Dimensional Cell Culture at the Frontiers of in Vitro Cancer Research: Present Perspectives

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ABSTRACT

In recent years, three-dimensional (3D) *in vitro* cell culture models have earned great attention, especially in the field of human cancer disease modelling research as they provide a promising alternative towards the conventional two-dimensional (2D) monolayer culture of cells with improved tissue organization. In 2D cell culture systems, the complexity of cells on a planar surface does not accurately reflects the *in vivo* cellular microenvironment. Cells propagated in 3D cell culture model, on the other hand, exhibit physiologically relevant cell-to-cell interactions and cell-to-extracellular matrix (ECM) interactions, important in maintaining a normal homeostasis and specificity of tissues. This review gives an overview on 2D models and their limitations, followed by 3D cell culture models, their advantages, drawbacks and challenges in present perspectives. The review also highlights the dissimilarities of 2D and 3D models and the applicability of 3D models in current cancer research.

Keywords: Cell culture, cellular microenvironment, cell-to-cell interaction, cellular morphology, cell polarity, extracellular matrix

INTRODUCTION

Till recently, cell culture systems that have experienced an accelerated growth are one of the most demanding scientific models available. This *in vitro* culture system is an indispensable tool for a wide spectrum of applications, ranging from research to industrial perspectives due to its adaptability to the experimental variations that are possible in such culture system (1). The cell culture techniques currently available have undergone a long way of revolution since late 1800s when Wilhelm Roux successfully maintains live neural plate cells from chick embryos in saline buffer for several days (2). Starting from early twentieth century, researchers attempt to develop cell lines by utilizing the cell culture techniques available at that time and a vast

collection of cell lines is finally blossomed in the mid-twentieth century since after the establishment of the HeLa, a first mammalian cell line in 1951. In fact, the establishment of cell lines and the advancement of cell culture techniques are interrelated. This is augmented by the fact that the growth of cell culture techniques is accompanied by the increase in the number of cell lines (1). However, the well-known two-dimensional (2D) cell culture system that was developed over a century ago has raises increasing doubts on its efficiency to maintain the structure and functionality of cultured cells, which is analogous to tissues (3). Thus, three-dimensional (3D) cultures were introduced to improve the fidelity and long term maintenance of cells in an *in vitro* environment.

2D Cell Cultures and Their Limitations

The 2D cell culture system is an *in vitro* static dish culture model that grows monolayer of adherent cells on a flat and rigid substrate, such as artificial plastic or glass, where cultured cells only in contact with each other at their periphery (4). This time-honoured 2D cell culture system is the most common and classically preferred conventional culture model to reconstitute the *in vivo* cellular microenvironment (5). Despite the fact that 2D culture models proven to play a pivotal role in biological research over many decades, a multitude of inadequacies and limitations associated have been increasingly recognized. Firstly, the ability of such culture system to emulate the *in vivo* conditions has been called into questions, because cells in the natural cellular environment are surrounded by extracellular matrix (ECM) and other cells in a complex 3D fashion (6). Secondly, the stress and artificial responses of cells experienced in 2D models due to the cell adaptation to the flat and rigid surfaces in order to establish a favourable environment for optimal cell growth, the morphological and/or functional features of cell *in vitro*, can significantly alter their ECM proteins expression (7, 8). Further, the setting of 2D models also promotes the uneven distribution of receptors on the cell surface and clustering which presumably affect the intercellular communication (8). Such adaptations of cells to the planar 2D culture system are inaccurately recapitulate of the *in vivo* environment as cells require significant morphological changes which will impair the cellular functions and metabolism to enable the survival in 2D cultures. Thirdly, the conventional 2D cell culture models lack of the metabolic gradients that present in the *in vivo* environment caused it to be unsuitable to represent the actual microenvironment, because movements of cells in the *in vivo* state follow a molecular gradient or chemical signal, important for majority of the biological processes, including cell differentiation, cell fate determination and signal transduction (9, 10). Taken together, these findings emphasize on the inadequacies of 2D models to accurately mimicry of the *in vivo* microenvironment. Consequently, these shortcomings of 2D models can diminish the cellular properties of 2Dly cultured cells such as the viability, proliferation, differentiation, general cell function and morphology, gene and protein expression and response to external stimuli and drug metabolism, allowing it to support only a limited levels of cell differentiation and *in vivo*-like functionality (11). As a result, it tends to give an unsatisfactory, misleading and sometimes misinterpreted data for *in vivo* responses and probably some important discoveries may be missed completely.

The poor predictive power of 2D cultures in preclinical cell-based drug and toxicity screening assays caused approximately 90% of promising preclinical drugs that passed *in vitro* preclinical studies fail in the subsequent clinical trials, and the failure rate is even higher in cancer drugs due to the unsatisfactory of clinical efficacy and undesired safety margin (12-16). Undeniable, immortalized tumour cell lines cultured in 2D models have contributed to the general understanding of tumour growth and progression. However, the inadequacy to effectively model tumour biology and with nearly 95% of drug candidate attrition rate, 2D culture model is regarded as a poor drug discovery model (17). In the most simplistic view, 2D culture systems imposed physiological constraints on cultured cells; whereby nutrients, oxygen or waste gradients are absent in the culture environment (4). Simultaneously, 2Dly cultured cells are forced to arrange into a monolayer morphology in a planar surface lead to the lacking of interactions between cells and their microenvironment that specifically important in nature tumour. Since 2D cell culture models are lack of realistic complexity and limited culturing of only single cell types, the delay in discovery of successful medical interventions is possible with the involvement of 2D models.

3D Culture Models and Their Potentials

Importantly, the 3D architecture of cellular microenvironment in the *in vivo* state allows cell-to-cell and cell-to-ECM interactions through biochemical and mechanical cues to establish a communication network capable to maintain the specificity and homeostasis of tissues. Over the past two decades, numerous 3D culture models have developed to overcome the shortfalls of 2D cell culture system. More recently, 3D cells culture models have gained increasing interest because they are capable to enhance the expression of differentiated function

and improved tissue organization that is not possible in 2D models (18). In comparison, 3D cell culture approach is a superior *in vitro* model over 2D cell culture models that takes into account of the spatial organization of cells. And thus, it allows intricate cell-to-cell interactions and cell-to-ECM interactions in all three dimensions with a dynamic transport system for nutrients, oxygen and discharge of waste products. Hence, cells are able to grow continuously in an undisturbed artificial environment, in contrast to 2D models where regular trypsinization is required even for normal cell growth. Evidently, 3D models have a greater physiological relevance than 2D models and this advantage bridges the gap between cellular physiology and the *in vitro* cell culture systems. In addition, the presence of intercellular communication in 3D models allow cultured cells to adopt a comparatively precise depiction of cell polarization. All in all, artificial 3D culture system is a better cell culture model compared to 2D culture model. The improved cellular interactions as in 3D models are reminiscent of the actual cellular microenvironment, and hence, the cellular responses and the behaviour of cells cultured in such *in vitro* setting are comparatively more reflective of the *in vivo* conditions (13, 19-21).

3Dly cultured cells possess morphological and/or functional features that are more accurately reflect the *in vivo* state (22). An emerging evidence demonstrated the cells grown in the 3D culture system mimic its natural shape and possess cellular heterogeneity similar to the *in vivo* counterparts. Moreover, 3Dly cultured cells display genotypes that significantly more relevant to *in vivo* state. Not only that, 3D model also allows the co-cultivation of multiple cell types and so it is more precisely imitates the natural *in vivo* microenvironment. Such 3D-based cell co-culture system is an ideal *in vitro* model that is useful in the discovery and understanding of the importance of intercellular communication in cell functions, particularly the role of stromal cells in tumour microenvironment (TME), which play a pivotal part in the development and progression of cancers (23). As evidenced by Weaver *et al.*, breast tumour cells upon the inhibition of β1-integrin are capable to revert to a normal epithelial phenotype, whether morphologically or functionally (16). Besides, 3D models have great stability and therefore, cell growth in 3D models are far more stable and have a longer lifespan (24). It was evident that cells cultured in 3D models can last at least up to 4 weeks, in turn, cells cultured in 2D models only last for almost a week due to cell confluency (4). Collectively, 3D model is undoubtedly a promising *in vitro* model, more appropriate to serves as an invaluable tool for the study of the long-term effects of candidate drugs. In addition, 3D model provides a more accurate representation of cytoarchitecture and till date, 3D models have been successfully allowed the study of more than 380 cell lines and extensively adopted in stem cell culture and differentiation, cancer cell biology, tissue engineering, cell-based analysis and drug discovery (1). The advancement of 3D models has resulted in significant acceleration of translation research in wide range of medical and cellular projects, including cancer biology, regenerative medicine and tissue engineering, and thus ultimately fuelled the development of novel drugs with low rate of attrition.

Strikingly, 3D-based culture of embryonic stem (ES) cells proved to promote the growth of self-organizing organoid of diverse tissues. Eiraku *et al.*, demonstrated the autonomous formation of the optic cup structure by 3D culture of mouse ES cells under serum-free floating culture of embryoid-body-like aggregates with quick reaggregation (SFEBq) with an addition of basement-membrane matrix component, Matrigel (25). The generated self-form ES-derived optic cup regulated in a spatiotemporally manner, possessed a distinctive apical-basal polarity and invagination pattern reminiscent of their *in vivo* counterparts. Suga *et al.*, have produced functioning pituitary tissue from mouse ES cells by 3D cell culture system under SFEBq condition (26). Moreover, the co-induction of hypothalamus and oral ectoderm within ES cell aggregate has successfully recapitulate embryonic pituitary development that shown highly layered structures as observed *in vivo* and the transplantation of the generated pituitary tissues has a notable capacity in restoration of the systemic glucocorticoid level in hypopituitary mice. Further, Sato and Clevers, have established a R-spondin-based 3D model that successfully grown a single intestinal stem cell, Lgr5-crypt base columnar (CBC) from mice into epithelial organoids with retained original identity (27). The *in vivo*-generated organoids have self-renewing capacity and cell-type composition closely reminiscent the *in vivo* state. Further, the engraftation of epithelial organoids into the colons of mice leads to the regeneration of epithelial patches that indifferent from the surrounding recipient epithelium. Other than that, human pluripotent stem cell-derived 3D organoid culture system by Lancaster and Knoblich, successfully developed into a cerebral organoid with various discrete but interdependent brain regions, recapitulate features and development of the human cortical development (28). Methods as demonstrated by Takebe *et al.*, for the generation of transplantable organ bud by co-cultivation of human induced pluripotent stem cells (HiPSCs) with endothelial cells and mesenchymal stem cells (MSCs) showed

a rapid vascularization and self-organization into functional and tissue-specific structure (19). It is a promising approach with therapeutic potency, in which the transplantation of *in-vitro*-derived 3D pancreatic condensate successfully treated type I fulminant diabetic mice. Overall, 3D culture system not only serves as a promising model for the study of biology and pathology, but it also a suitable system for the recapitulation of embryonic development and disease that is difficult to recapitulate in mice, for example, microcephaly, possible facilitates the realization of generative medicine and/or regenerative therapies for organ defects.

Comparision between 2D And 3D Cell Culture Models

2D models generally grow only single type of cells that forced into a monolayer morphology on a flat surface, such as glass or tissue culture polystyrene plastic flasks (Figure 1A). In contrast, 3D models allow the growth of either single type of cells or co-cultures on pre-coated scaffold/matrix (Figure 1B) or as 3D aggregates or spheroids within a scaffold/matrix (Figure 1C) or suspended in medium containing matrix in a scaffold-free manner (Figure 1D). The attachment and spreading of cells in 2D models are normally occur within minutes due to its restraint-free nature. In turn, proteolytic degradation of physical environment is necessary in 3D models prior to the attachment and spreading of cells, which can occur in hours or even days (30). In planar 2D models, cell attachment occurs at only one side of the cell, forcing cells to have limited cellular contact and interactions; whereas cell attachment in 3D models occurs around the entire surface of the cells (31). Such setting of 3D cell culture models allows the existence of cell-to-cell interactions and cell-to-ECM interactions and hence enable the crosstalk between cultured cells and their surrounding 3D environment as in the *in vivo* conditions. In fact, the interactions that existed in 3D culture models contributed to the fundamental differences as observed in 2D and 3D models. These interactions are important during *in vitro* culturing to achieve the *in vivo*-like structural organization and connectivity, able to limit or even diminish cell morphology and functional features of cell, including cell viability, differentiation, proliferation, gene and protein expression (6).

Regardless of the cell line and matrix dependence, the rate of proliferation of 2Dly and 3Dly cultured cells are usually differed, mainly due to their dissimilarity in spatial organization. Moreover, a tremendous amounts of cell line cultured in 3D models exhibit a reduced proliferation rate in comparison to those 2Dly cultured cell lines (32-36). Generally, the proliferation rate of 3Dly cultured cells is more accurately mimicry of the rate of growth of cells in the *in vivo* environment than the 2Dly cultured cells (34). Besides, the dissimilarity in cell morphology is obvious when comparing 2D and 3D cultures. In fact, appropriate cell polarity is another important physiological attribute conferred by the cellular interaction, including cell-to-cell and cell-to-ECM interactions that possible in 3D models. With the absence of cellular contact in unnatural 2D models, it resulted in the default apical-basal polarity and alteration of cellular morphology that ultimately influence the cellular function (31). The cell morphology such as cytoskeletal organization and cell adhesions of 3D cultures are shown to be much more closely resemble to their *in vivo* counterparts due to the relatively precise cell polarization, compared to 2D cultures with only partial polarization. In addition, the setting of 2D models created a non-physiologically uniform environment, where uniformly rich oxygen, nutrients and growth factors are provided to all cells in the culture (37). Unlike conventional 2D monolayer cell culture models, oxygen, nutrients and waste gradients are present in the 3D models. Moreover, the restricted nutrition and oxygenation environment encountered by 3Dly cultured cells are actually emulate the microenvironment as in the *in vivo* tissues (38). With similar physiological environment of nutrients and gaseous exchange restriction, the cellular heterogeneity present in 3D models resembles to the *in vivo* tissues, where both comprises of cells at various stage, including proliferating, apoptotic, necrotic, quiescent and hypoxic cells (39, 40). In contrast, cellular heterogeneity is absent in 2D models and this system is generally composed of only flat and stretched cells that exhibit a relative uniform proliferation across the surface due to the consistency of medium exposure (6). These differences strongly suggested that 3D model is an excellent model system that able to supports varying degrees of cell complexity and functionality as in the natural environment compared to 2D model. The generation of microenvironment that mimic the physiological conditions enable the cellular response of 3Dly cultured cells to drug treatments to be more reflective of the *in vivo* conditions (41-43).

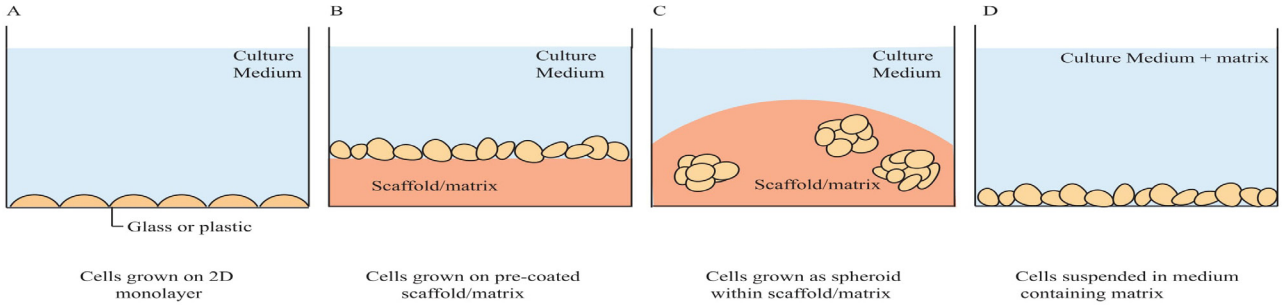

Figure 1: Schematic diagram of two-dimensional (2D) cell culture (A) and three-dimensional (3D) cell culture systems: cells grown on pre-coated scaffold//matrix (B), grown as spheroid within scaffold/matrix (C) and scaffold/matrix-fee manner in medium containing matrix

Table 1: Two-dimensional (2D) versus three-dimensional (3D) cell culture systems

Variables	2D	3D
Morphology	Flat and stretched cells forced into a monolayer on a flat surface (6).	Cells maintain its normal shape and structure into a 3D aggregates or spheroids (7).
Cell attachment	Normally occur within minutes at only one side of the cell (30).	Can occur in hours or even days around the entire surface of the cells (30).
Proliferation rate	Cells proliferate at a relatively uniform rate but often at a faster rate than *in vivo (6).*	Accurate representation of cell growth rate *in vivo* (34).
Cell polarity	Cells displayed a default apical-basal polarity with only partial polarization (4, 31).	Precise depiction of cell polarization (4).
Cellular heterogeneity	Cells generally in the same stage (6).	Spheroids comprises of cells in various stage, such as quiescent, proliferating, apoptotic, necrotic and hypoxic cells (39, 40).
Cell culture environment	Non-physiologically uniform environment provides rich oxygen, nutrients and growth factors to all cells uniformly (37).	Restricted oxygenation, nutrition environment and waste gradient are accurately emulate the natural cellular environment (38).

Applications Of 3D Cell Culture Model in Cancer Research

3D culture models have been a subject of interest since it exhibits a hierarchical structure and cellular heterogeneity, enable a more precise representation of *in vivo* cell morphology and function. With this advantage, 3D models emerge as a suitable model in revealing of novel and unanticipated visions into tumorigenesis mechanism, significantly accelerate the research in cancer biology. Frequently, 3D culture model is used extensively in studying of the expression of hallmark characteristics of tumour cells, including uncontrolled proliferation, expression of growth signals, transition of cells, anti-apoptosis, invasion, angiogenesis and metastasis (44). 2D cell culture models genuinely contributed to a multitude of knowledge in cancer biology, however, the inability of 2Dly cultured cells to form a multidimensional structure simply do not reflects the *in vivo* cellular microenvironment. In comparison, 3D cultures of tumour cells with customized microenvironments are a scientifically-rigorous culture system that better mimic the cells growing within the living tumours.

Myungjin Lee *et al.*, cultured 31 epithelial ovarian cancer (EOC) cell lines in 3D by using polyhydroethylamethacrylate-coated tissue culture plastics for 14 days (45). The 3Dly cultured EOC cell lines formed multicellular aggregate structures (MCA) and restored the histological differentiation of the primary tumours that was absent in 2D cultures. Moreover, the molecular changes in 3Dly cultured EOC cell lines much more closely reflect the molecular features of the primary tumours compared to 2Dly cultured cells. Whilst, Jaganathan *et al.*, have developed an *in vitro* 3D heterogeneous tumour model for breast cancer by utilising magnetic levitation system in order to recapitulate the *in vivo* breast cancer cell-fibroblast interactions (46). The proposed 3D co-culture model proves to be a useful model in dissection of the influences of tumour microenvironment on tumour cell behaviours. It is said to be an advantageous model due to its capability

to form a larger sized breast tumour model in short time, manipulation on the tumour compositions and densities, and more accurately resemble the *in vivo* tumour microenvironment, and thus it allows a better understanding of tumour biology. Besides, the 3D co-culture model comprises ameloblastoma-associated fibroblasts (AAFs) and gingival fibroblasts (GFs) with head and neck squamous cell carcinoma cell lines separately developed by Chantravekin and Koontongkaew, provided a valuable tool to reveal the tendency of AAFs in stimulate the proliferation and invasion of tumour cells more than GFs, putatively through TGF-β expression (47). And, the 3D laminin rich ECM models by Cichon *et al.*, further embark on the importance of cell-ECM interactions in cellular function (48). 3Dly cultured lung cancer cells displayed an observable development of multicellular structures that reflective of the phenotypic alterations controlling of the cancer cell malignancy.

Furthermore, the physiological relevance of 3D culture model enable it to be a representative of a promising *in vitro* cell culture system for the study of cancer-relevant patterns of cellular processes. By using 3D culture models, Harma *et al.*, successfully analysed the growth modes, migration and invasion of prostate cancer cells, because the nature of 3D models allowed the monitoring and modulation of invasive processes of tumour cells in organotypic environment (49). In addition, it is evident that 3Dly cultured prostate cancer cells are more relevant to the tumour cell biology than those cultured in 2D model, enable it to make a significant contribution to the finding of polarized epithelial structures which resemble the morphology, biochemistry and invasive processes of tumours as in the *in vivo* conditions. Bokhari *et al.*, have demonstrated the capability of 3D models in narrowing the gap between *in vitro* cell culture systems and the cellular physiology (24). The HepG2 liver cells cultured in 3D model shown to exhibits a greater viability, maintained structural integrity and less susceptible to apoptosis or necrosis even at a high level of cytotoxin compared to their 2D counterparts. Moreover, HepG2 cells grown on 3D model performed a normal metabolic activity and cultured cells occupied a 3D environment allowed them to interact with adjacent the cells in order to maximise their surface area as in the natural cellular environment. In contrast, HepG2 cells displayed a heterogeneous and disorganised appearance with flat extended structures on 2D substrate, and appeared unhealthy with some of the cells rounding up and some disintegrating starting from day 14. In essence, these findings embrace the potentials of 3D model to arise as an excellent model system that resemble more closely of the *in vivo* microenvironment.

Applications in Oral Cancer

2D models are a valuable tool in oral cancer research that have contributed a tremendous amount of knowledge underlying the mechanism of cancer, however, the physical and biochemical features of the solid tumour mass do not achieve in such monolayer cultures. Owing to the limitations of 2D models, 3D models appear to be a promising alternative that bridge the gap between 2D and animal studies. At present, the 3D models of normal oral mucosa are well established and the co-culture model developed has been adapted by researchers to recapitulate oral dysplasia or cancer (50, 51).

The bidirectional interactions between cells and their microenvironment are important for the normal tissue homeostasis as well as tumour growth (52). In native tumours, the communication between tumour cells and the associated stroma in TME is achieved via cell junctions, receptors, hormones or soluble factors are critical in sustaining the tumour growth, invasion and metastasis. Li *et al.*, have demonstrated the role of cancer-associated fibroblasts (CAFs) in promoting the growth, proliferation, mobility, invasion and epithelial–mesenchymal transition (EMT) of oral tongue squamous cancer cell lines (OTSCC) in a 3D co-culture model (53). The model provided the ability to dissect the intercellular communication between CAFs and OTSCC cell lines in order to determine the factors manipulating the behaviour of OTSCC.

It is well known that the crosstalk between the stromal microenvironment and the tumour cells plays a significant role in activation of the signalling pathways that can promote tumour invasion and progression. In order to examine the feasibility of 3D models in studying oral cancer cell invasion, Duong *et al.*, developed a 3D construct that included an epithelial component of oral squamous carcinoma cells (OSCC) that seeded atop of a layer of connective tissue containing oral mucosa fibroblasts, separated by a reconstituted basement membrane. By utilizing the constructed 3D models, the invasion of the OSCCs into the underlying basement membrane and the connective tissue stroma, as well as their associated mechanism can be observed in varying culture conditions and treatments at different time intervals with ease (54). Whilst, the 3D human oral squamous cell carcinoma (OSCC-3) model using synthetic poly(lactide-co-glycolide) (PLG) scaffolds established by Fischbach *et al.*, (55) able to restored the histological characteristics of primary tumours. Besides, 3D PLG cultured OSCC-3 cells transitioned to a fibroblastic morphology, acquired migratory phenotypes and upregulated mesenchymal biomarkers suggests that tumour cells cultured in 3D PLG microenvironment exhibit

enhanced invasive potential. And, the increased concentration of fibronectin and enhanced human umbilical vein endothelial cell (HUVEC) proliferation in 3D PLG culture of OSCC-3 cells linked to their enhanced angiogenic capacity in relative to their 2D counterparts. Overall, 3D PLG culture enhanced the invasion capacity and tumour progression of OSCC-3 cells.

In the study conducted by Eriksson *et al.*, aimed to examine the effect of co-culture environment on ameloblastoma (AM-1) cells within a collagen construct, the AM-1 construct and the bone-like construct composed of human osteosarcoma (HOS) cells and Bio-Oss granules within a collagen construct was joined together with an acellular support gel (56). The established co-culture model demonstrated the presence of HOS cells in attracting the migration of AM-1 cells through the bone-like part of the construct is then further quantified by Transwell inserts. Further, the presence of HOS cells in the co-culture construct upregulated the RANKL expression of AM-1 cells, but the presence of AM-1 cells downregulated the OPG and NF-κB expression of bone-like construct increases the rate of bone resorption. In order to analyse the role of neutrophils in invasion potential of OSCC, Glogauer *et al.*, established a direct and indirect co-cultures of human peripheral blood neutrophils and UMSCC47 cells (OSCC cell line) in Matrigel-coated Transwell support (57). The established co-cultures model revealed that the presence of neutrophils increases the invasiveness of UMSCC47 cells even without direct contact via the activation of invadopodia formation and matrix degradation to enable the oral cancer cells to invade into the surrounding tissues.

Drawbacks of 3D Model

On the downside, even the most impressive and advanced 3D models fail to exactly recapitulate the morphological characteristics including tissue-tissue interfaces, spatiotemporal gradients of oxygen, nutrients and chemicals, and the mechanically active microenvironmental cues of a living organ that are important for their general function (58). Scaffold/matrix-based 3D culture systems possess a major technical challenge in terms of the scaffolds/matrix used in the system. Of the various 3D models developed, some uses matrices of animal origin components which may lead to the implementation difficulties in clinical work, whereas some uses matrices made from tissue such as basement membrane extracts and these matrices potentially contain unknown or undesired components, such as growth factors and viruses. Other than that, some matrices that allow for cell attachment in culture system may not be able to effectively remove the cells attached on the matrices and thus it affects the assay development (59). As example, collagen hydrogels and Matrigel have to be handled well to maintain its density and low viscosity respectively in order to allow the manipulation and mixing of cells in 3D culture system. Hence, the choice of scaffolds or matrix, taking into the consideration of porosity, pore distribution and exposed surface area of the scaffolds play a critical role in the distribution and penetration of cells within the scaffold volume, which will significantly affect the architecture of the generated ECM. Whilst, the 3D cell cultures in gels must be control with caution, because the culture conditions, including the culture pH and temperature are very important for the functional and effectiveness of a model. These technical difficulties embark on the user-unfriendly nature of the 3D model. Besides, there is a poor level of reproducibility and consistency between sets of experiment and difficulties in cell extraction from the bio-scaffold for analysis due to the increased size and tortuosity of the scaffold. Also, the creation of 3D cell construct can be very difficult and laborious due to the requisition of many different components. Undoubtedly, 3D model provided a more accurate system for the generation of microenvironment that mimic physiological conditions, however, it has a limited capacity in scaling of cell culture system and also the post-culturing processing of the system. In accordance to the size of the bio-scaffold and transparency of material used, the imaging of 3D model may be difficult depends on the microscope depth. It is noted that the frequent use of 3D culture models in different research areas is generally followed by the technical challenges in terms of sensitivity, performance, and compatibility of 3D models with high-throughput screening instruments. However, the assays currently available for the investigation of cellular responses to drug interactions, including cell-to-cell and cell-to-matrix interactions, cell migration and dose dependent cell viability are not optimized for the increasingly sophisticated 3D models. Therefore, optimization is highly required for specifically prepared 3D culture systems to suit for the most of the experimental approaches (4).

Challenges of 3D Model

Even though 3D models are useful in reconstitute the actual *in vivo* cellular environment, in which cell-based experiments can be performed very accurately, 3D models have yet to replace the traditional 2D models on a large scale. The limitations of existing 3D culture models in terms of its scalability, sensitivity, reproductivity, and compatibility with the high-

throughput screening instruments, remained as major challenge for 3D model. Other than that, 3D models also faced challenges on limited data that addressed the mechanism of cell differentiation, drug interaction, and cell signalling. Therefore, the limited knowledge on the functionality of 3D models diminishes the confidence for adoption, although number of publications is increasing rapidly. Further, 3D model also encountered challenges in designing different experimental assays with distinct characteristics for different cell types or nature of applications. Hence, a universal standardized 3D culture system with systematic optimization and characterization is strongly necessary to fully utilized the benefits of 3D culture models in the understanding of the *in vivo* cellular behaviours which ultimately facilitates the development of various biological research.

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