Modeling nasopharyngeal carcinoma in three dimensions (Review)

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Abstract. Nasopharyngeal carcinoma (NPC) is a type of cancer endemic in Asia, including Malaysia, Southern China, Hong Kong and Taiwan. Treatment resistance, particularly in recurring cases, remains a challenge. Thus, studies to develop novel therapeutic agents are important. Potential therapeutic compounds may be effectively examined using two-dimensional (2D) cell culture models, three-dimensional (3D) spheroid models or *in vivo* animal models. The majority of drug assessments for cancers, including for NPC, are currently performed with 2D cell culture models. This model offers economical and high-throughput screening advantages. However, 2D cell culture models cannot recapitulate the architecture and the microenvironment of a tumor. *In vivo* models may recapitulate certain architectural and microenvironmental conditions of a tumor, however, these are not feasible for the screening of large numbers of compounds. By contrast, 3D spheroid models may be able to recapitulate a physiological microenvironment not observed in 2D cell culture models, in addition to avoiding the impediments of *in vivo* animal models. Thus, the 3D spheroid model offers a more representative model for the study of NPC growth, invasion and drug response, which may be cost-effective without forgoing quality.

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Contents

1. Introduction

Nasopharyngeal carcinoma (NPC) is a type of cancer that affects the nasopharynx, commonly at the posterior and superior region in the fossa of Rosenmüller (1). It is a geographically distinct cancer, which is prevalent in south-east Asia, southern China and southern Africa (2). Viral, dietary, hereditary and lifestyle factors have been identified as risk factors for NPC (2). Current NPC treatment consists of radiotherapy, chemotherapy or chemo-radiotherapy; treatment resistance, particularly in advanced and recurrent cases of NPC, remains a challenge (2). NPC is staged according to the TNM system, whereby T describes the primary tumor invasion to the tissue or organs near the nasopharynx, N describes the spread to the lymph nodes and M indicates the metastasis of the tumor. NPC is usually detected at a late stage (III or IV) (2). Early-stage NPC has unspecific and ambiguous clinical symptoms such as neck lumps, bloodstained sputum, mild hearing loss and a unilateral headache which may be ignored by its sufferer or misdiagnosed by a doctor (3). Ultimately, this leads to a late disease presentation as well as detection. The pathogenesis of NPC involves genetic and epigenetic changes in the nasopharyngeal epithelium (4). Previous studies have improved current understanding of the potential molecular targets and signaling pathways involved in NPC pathogenesis, which has assisted the development of targeted therapies for the treatment of NPC, including cetuximab (Erbitux®), bevacizumab (Avastin®), pazopanib (Votrient®), the phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) dual inhibitor PF-04691502 and apogossypolone (5-9).

Potential therapeutic compounds are typically evaluated using two platforms: Two-dimensional (2D) cell culture models and *in vivo* animal models (10). However, the 2D model does not epitomize the microenvironment and architecture of a tumor *in vivo*, whereas experiments involving animal models are expensive, time-consuming and often include intricate surgeries. The 3D spheroid model may balance the cost-effectiveness of 2D models and the physiological complexity of animal models as the 3D model more closely recapitulates *in vivo* conditions of a tumor. The tumor phenotype, architecture and interaction with its microenvironment are crucial for determining the response and resistance of tumors to specific drug treatments (11,12). In this review, the ethics, costs and practical considerations, including experimental procedures, are discussed, in addition to the biology of the models. This review will examine the advantages and briefly discuss the disadvantages that 3D spheroid models may provide for the study of NPC, as compared with 2D models and *in vivo* models.

2. 2D cell culture models

The 2D cell culture model involves the culturing of cells in a plastic dish, which allows for the formation of a monolayer of cells adherent to the surface of the dish (13). 2D cultures are cost effective, simple to establish and easy to maintain (12). This ease of use facilitates the high-throughput screening of potential therapeutic agents.

The cells grown in this model have direct contact with the microenvironment and thus, maximum exposure to available nutrients and growth factors (14) (Fig. 1). The cells may adapt to the artificial growth conditions and upregulate certain growth-associated genes that promote cell proliferation (15). However, this model does not recapitulate the conditions of an *in vivo* tumor, which is composed of heterogeneous cell types with established concentration gradients and may have sub-maximal drug penetration (13,16). Cells growing in a single monolayer format do not exhibit communication such as the cell-matrix crosstalk observed in three-dimensional microenvironment (Fig. 2), whereby the cells in three-dimensional cultures are in constant cross-talk with the stroma and other cells, including fibroblasts, immune cells and endothelial cells. The reactions of monolayer cultures to external stimuli often do not reflect a physiological effect, as demonstrated by trials investigating the administration of chemotherapeutic drugs, wherein drug sensitivity results obtained from monolayer cultures have been shown to be misleading and non-predictive for the *in vivo* trials because 2D cell culture models do not epitomize the tumor microenvironment and architecture that is present *in vivo*. These features of the 2D model impede the evaluation of drug penetration and drug resistance (15,16).

In studies of NPC, 2D cell culture models are often utilized for various drug sensitivity assays (17-19). These usually demonstrate effective reactions in the micromolar or nanomolar ranges. Whether the results obtained from 2D cultures may be recapitulated in *in vivo* models requires further study, as the critical components of the *in vivo* tumor microenvironment are not present in the 2D model.

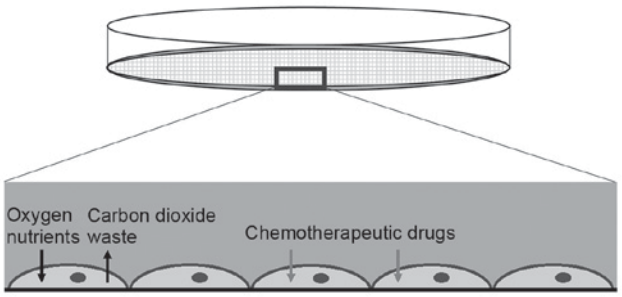

Figure 1. 2D cell culture model. Cells grown in 2D cell culture adhere to the plastic surface of the plate. They receive maximum exposure to drugs with optimal diffusion of nutrients and waste products. The 2D culture model provides the cells with the optimal conditions to proliferate, thus, inducing high proliferative rates, although this does not reflect the tumor behavior *in vivo*. 2D, two-dimensional.

3. *In vivo* animal models

Following identification of a potential therapeutic agent, it must be further examined in models that are more physiologically relevant to humans, usually an *in vivo* animal model and/or 3D cell culture model. The *in vivo* animal model may use mammals, including mice, rats, rabbits or monkeys; zebra fish or other animals are also viable models (12,20). Mouse models utilized for drug testing often involve the use of tumor transplantations such as syngeneic models, subcutaneous xenografts or orthotopic xenograft of cell lines and patient-derived tumor xenograft (PDTX) models (21,22). The syngeneic models are produced by allografting tumors from an animal into another animal that is genetically similar or of an identical strain. Subcutaneous xenografts involve the injection of cancer cells into the subcutaneous tissue of the mice, while orthotopic xenografts involve the injection of cancer cells according to the respective location of the cancer type. Both are artificial in comparison to genetically engineered mouse models, wherein the tumors may occur spontaneously at their natural site (23,24). PDTX models are established by directly engrafting freshly isolated tumors from patients into immunodeficient mice (22,25).

The xenograft model allows human cancer cells to directly interact with the murine stroma, including lymphatic and blood vessels, and therefore facilitates investigation of the growth behavior and drug responses of human cancer cells *in vivo* (12). Furthermore, as standardized techniques are used (the same number, passage and culture conditions of injected cells), this model provides improved control over the timing of tumor growth and the time points of drug administration, which enable the collection of reproducible data (12).

In addition to xenograft models for NPC (26,27), an orthotopic model for NPC was also developed by luciferase-tagging of C666-1 and HONE-1 NPC cells and injecting them into the nasopharyngeal epithelium of immunodeficient NOD. Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ mice (28). It was identified that, in a more natural microenvironment, the tumor cells exhibited characteristics that more accurately mimicked the metastatic and invasive human NPC, particularly during metastasis to numerous distant sites (bone, lung, liver). Therefore, this model may have advantages as a tool for the investigation of potential therapeutic agents for advanced NPC (28). Treatment with an

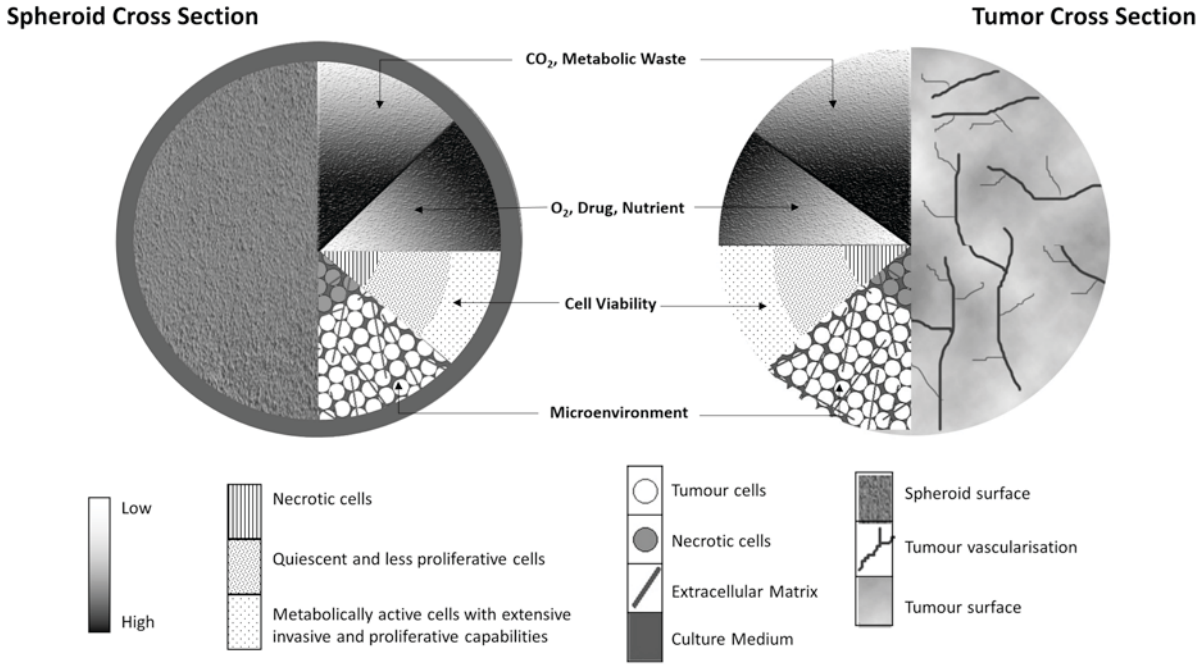

Figure 2. Similarities between the 3D spheroid model and a tumor *in vivo*. The spheroid received nutrients from the medium and the tumor through its vascularization, although they are subject to concentration gradients, which limit efficient diffusion. This creates a microenvironment, leading to various conditions of cells within the tumors and spheroids. The necrotic core exists due to the lack of available nutrients and the accumulation of waste, whereas the increased metabolic activity at the periphery is due to the more efficient diffusion of nutrients and waste. In each condition, the cells exist in a 3D conformation with interactions with the extracellular matrix. 3D, three-dimensional.

mTOR inhibitor and serolimus was observed to significantly inhibit the growth and metastasis of NPC tumors, suggesting that the PI3K/protein kinase B (AKT)/mTOR signaling pathway is a potential therapeutic target for the treatment of NPC (28).

Xenograft models offer a variety of benefits in drug sensitivity studies; however, they have their own disadvantages. The stromal component is of murine origin and, thus, reflects the microenvironment of mice and not humans (29). Furthermore, drug study data from xenograft models are not always similar to the data obtained in clinical trials (29,30). Cell lines used to establish xenografts (with the exception of PDTXs) are subjected to strong selection for defective apoptosis, are not cultured in their natural environment and are implanted into a host that does not have an intact immune system (12). Orthotopic xenograft models require intricate surgery to implant the tumor cells at specific sites, including the brain, liver, kidney and nasopharynx, or they may not be representative of the *in vivo* behavior of tumor cells in metastasis and invasion, which is fundamental for therapeutic studies (28). These experiments are time consuming (30) and are subject to the regulations of animal ethics (12).

4. 3D spheroid models

The 3D spheroid model was first created in 1970 (31) to study the mechanisms underlying tumorigenesis, including cell proliferation, invasion and metastasis (32). This model is based upon the physiological manner of culturing cells in their natural 3D state, where the cells aggregate to form multicellular or sphere-like structures, aided by artificial extracellular matrix (ECM) (11). 3D spheroids maintain their structural integrity, which is a key component for mimicking the conditions of a tumor *in vivo*, in addition to the presence of the ECM (33,34). Ideally, the 3D spheroid model serves to provide a balance between 2D culture and *in vivo* animal approaches. 3D spheroids may be used as simple cancer cell line spheroids, co-cultures of cancer cells with other cell types (e.g. fibroblasts) within the spheroid (35) or as a culture for the maintenance of patient tissues (36).

A key characteristic of the 3D spheroid is that it recapitulates the *in vivo* tumor microenvironment and architecture (37) (Fig. 2). Three-dimensional spheroids grow suspended in a liquid medium, which supplies nutrients and facilitates gas exchange and waste disposal (38). Due to its structure, a gradient of nutrients and oxygen forms as cells go deeper into the tumors. Peripheral cells which are closer to the vasculature have maximal exposure to nutrients, oxygen and chemotherapeutic agents. Due to poor vascularization within solid tumors, concentration gradients of oxygen, nutrients and metabolic wastes are established (39). The shortage of oxygen and nutrients also creates hypoxic regions in the center of the tumor and cells in these regions often exhibit drug resistance (40), as drugs are often aimed to target rapidly growing cells and some are inefficient in the acidic microenvironments (39).

The 3D spheroid model may also be utilized to investigate drug bioavailability in addition to sensitivity. Melanoma spheroids treated with the B-cell lymphoma 2 homology domain 3-mimetic ABT-737 after seven days of growth demonstrated that spheroid peripheral cells were responsive to the drug

Table I. Comparison of the techniques of spheroid culture, associated with cell type, cell density, method of culture, type of base layer and the medium cocktail.

Cells	Cell density	Method	Base layer	Medium cocktail	References
HONE-1	$3x10^3$/NA	Liquid overlay method	Matrigel	RPMI + 2% Matrigel	(8)
C666-1	$5x10^3$/96 well	Hanging drop	No	DMEM/F12 serum free media + 20 ng/ml EGF, 20 ng/ml FGF and 20 ng/ml IGF	(42)
HONE-1 HK1	$1.3x10^4$/NA $1.7x10^5$/NA	Drug screening method	No	IMDM with 10% serum	(43)
TW01	5x104/10 cm plate	Ultra Low attachment plates	No	DMEM with 4% serum	(67)
HONE-1	$1x10^4$/48 well	Liquid overlay method	Matrigel	RPMI + 2% Matrigel	(73)
C666-1	$2x10^3$/24 well	Ultra Low attachment plates	No	DMEM/F12 serum free media + 20 ng/ml EGF, 20 ng/ml bFGF and 20 ng/ml insulin	(74)
C666-1, CNE2	$1x10^6$/T25	Ultra-Low attachment plates	No	DMEM/F12 serum free media + 20 ng/ml EGF + 20 ng/ml bFGF + 2% B27 and 1% penicilin-streptomycin	(75)
C666-1	$2x10^3$/24 well	Ultra-Low attachment plates	No	DMEM/F12 serum free media + 20 ng/ml EGF, 20 ng/ml bFGF and 20 ng/ml IGF	(76)
SUNE-1, CNE2	$1x10^4$/24 well	Liquid overlay method	Matrigel	RPMI with 5% serum	(77)
CNE2	NA	Liquid overlay method	Agar	RPMI	(78)
Xeno-C15	$1x10^5$/96 well	Ultra-Low attachment plates	No	RPMI with 5% serum	(79)
Xeno-C15	NA	Ultra-Low attachment plates	No	RPMI with 7.5% serum	(80)
C666-1	$1x10^6$/24 well	Ultra-Low attachment plate	No	DMEM/F12 serum free media	(81)
TW03	$1x10^2$/NA	Liquid overlay method	Agar	IMDM with 1% serum	(82)
CNE2	NA	Liquid overlay method	Agar	NA	(83)
HONE-1	$4x10^3$/NA	Liquid overlay method	Matrigel	DMEM with 10% serum	(85)
C666-1	$1x10^3$/24 well	Ultra-Low attachment plates	No	DMEM/F12 serum free media	(86)
NP69	$5x10^4$/NA	Liquid overlay method	Collagen	MCDB151 low calcium (0.1 mM) media with 1% serum + other growth supplements	(87)
CNE2	NA	Liquid overlay method	Agar	NA	(88)
C666-1	$1x10^3$/24 well	Ultra-Low attachment plates	No	DMEM/F12 serum free media + 20 ng/ml EGF +10 ng/ml bFGF + 0.4% BSA and 2% B27	(89)
HONE-1	$0.5x10^2$-$1.5x10^2$/T25	Liquid overlay method	Gelatin	DMEM with 10% serum	(90)
TW01	$1x10^3$/96 well	Liquid overlay method	Agar	DMEM with 10% serum	(91)
CNE2	$2x10^2$/NA	Liquid overlay method	Matrigel	RPMI with 10% serum	(92)
TW01, TW06	$2x10^4$/6 well	Liquid overlay method	Agar (0.3%)	DMEM with 10% serum	(93)
CNE2	$2x10^4$/NA	Ultra-low attachment plates	No	DMEM/F12 serum free media + 10 ng/ml EGF, 10 ng/ml FGF and B27	(94)

Table I. Continued.

Cells	Cell density	Method	Base layer	Medium cocktail	References
C666-1 HONE-1 HK1	$1x10^5$/24 well $4x10^4$/24 well $4x10^4$/24 well	Ultra-low attachment plates	No	RPMI with 10% serum	(95)
CNE1, CNE2	$1x10^3$/6 well	Ultra-Low attachment plates	No	DMEM/F12 serum free media + 20 ng/ml PDGF + 100 ng/ml EFG + 1% N2, 2% B27 and 1% antimycotic	(96)
TW02, TW04	$5x10^2$/24 well	Liquid overlay method	Agar	DMEM serum free media + 20 ng/ml EGF and 20 ng/ml bFGF	(97)
CNE2	NA	Liquid overlay method	Agar	NA	(84)
CNE2	NA	Liquid overlay method	Agar	NA	(98)
HK1	$5x10^3$/96 well	Liquid overlay method	Agar (1.5%)	RPMI with 10% serum	Lian *et al*., (unpublished)
C666-1	$1x10^4$/96 well	Liquid overlay method	Agar	DMEM/F12 serum free media + 10 ng/ml EGF and 10 ng/ml bFGF	(unpublished)

NA, information not available; IMDM, Iscove's modified Dulbecco's medium; DMEM, Dulbecco's modified Eagle's medium; F12, Ham's F-12 nutrient mixture; MCDB151, molecular, cellular, and developmental biology 151 medium; B27, B-27® supplement serum-free; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; IGF, insulin-like growth factor; FGF, fibroblast growth factor.

and that core cells were not, indicating that the drug did not diffuse effectively into large tumors (41). This observation was concordant with another study, which used a CXCL8 peptide inhibitor, termed Ac-RRWWCR-NH2, the C-X-C motif chemokine receptor 2 specific inhibitor SB225002 and the PI3K/AKT inhibitor LY294002 to target chemokines in NPC C666-1 spheroids (42). HONE-1 spheroids, which were used to investigate the effects of lapatinib, a dual tyrosine kinase inhibitor against epithelial growth factor receptor and human epidermal growth factor receptor 2, also exhibited similar drug diffusion problems (43).

The ECM is another feature that affects the biomimetic capacity of the 3D spheroid model. Under *in vivo* conditions, tumor cells establish biological crosstalk with the ECM (44). This interaction with the ECM may determine the level of cancer cell activity, including aggressiveness (45). Cells secrete various components, or induce other cells to secrete soluble factors, in a paracrine manner, including matrix metalloproteinases (MMPs), which allow cancer cells to penetrate the matrix, invade and metastasize (46-48). The ECM components secreted by spheroids include fibronectin, collagen, laminin and glycosaminoglycan (49-51). Each of these proteins has specific effects on the progression of cancer; laminin, for example, has a role in cancer invasion and metastasis (52).

Following their formation, 3D spheroids must be embedded in a matrix to enable them to proliferate and invade as tumors *in vivo*. The matrix consists of a scaffold, a buffering system, and nutrients, prepared using components that recapitulate the *in vivo* physiology. Typical components used include Eagle's minimal essential medium, L-glutamine, fetal bovine serum, sodium bicarbonate and Cultrex® Bovine Collagen I (Trevigen Inc., Gaithersburg, MD, USA) (53,54). Collagen type I is a major ECM protein of human organs and is available in abundance as a reagent with Cultrex® Bovine Collagen I (55). This component of the 3D spheroid ECM provides a cell scaffold, a structure for nutrient delivery and a matrix for cell signaling whilst having the advantage of being easily manipulated for the required tensional force and elastic modulus (53). The components used are variable and are available commercially (Matrigel and hydrogels), and these have been reviewed extensively in previous studies (14,56-58). The components used to prepare the polymers, the polymer concentration and the polymerization conditions affect the interactions and phenotype of the spheroid; therefore, consistency in the method of polymer preparation is crucial (59). Matrix stiffness has been previously demonstrated to affect cell invasion, in addition to the expression of MMPs (60,61).

The 3D spheroid model offers a more ethical approach to the screening of therapeutic agents as no animal sacrifice or surgery is required. The model also allows freedom to use reagents that recapitulate components that closely mimic humans, such as the collagen type I rather than the murine system. This may confer an advantage as the 3D model is intended to approximate the microenvironment of human tumors. Furthermore, the cells may be observed and quantified with imaging equipment, staining dyes and software in real time (41,62). Utilizing the 3D spheroid model is also cost-effective and efficient, as high-throughput drug sensitivity screening may be conducted, as compared with animal studies in which they cannot (12). Therefore, the 3D model

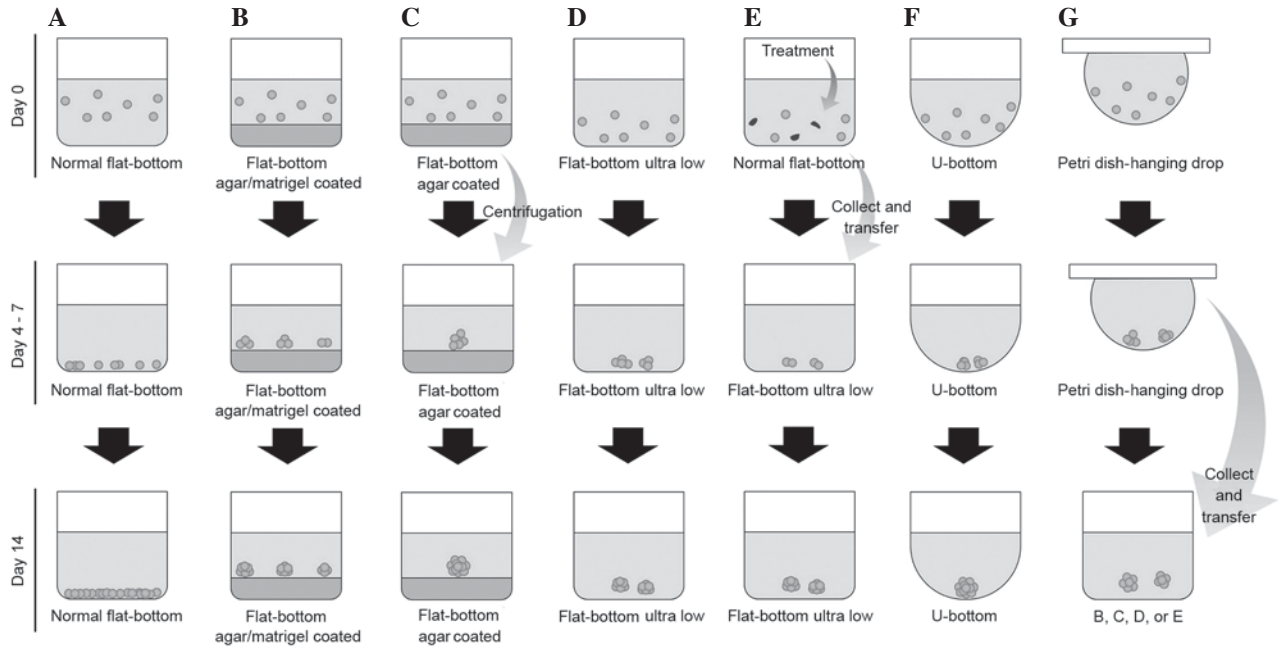

Figure 3. Comparison between various methods and plate types used for the generation of 3D spheroids. (A) Normal flat-bottom cell culture plate; (B) Normal flat-bottom cell culture plate coated with agarose or Matrigel; (C) Normal flat-bottom cell culture plate coated with agarose; the cells clump due to centrifugation; (D) Ultra-Low attachment plate; (E) Modification of drug resistant spheroid: drug treatment is conducted in a ultra-low attachment plate prior to spheroid culture; (F) U-bottom plate; (G) Hanging drop method using a petri dish: following the formation of cell clumps, the aggregates may be transferred into plate B, C, D or E. 3D, three-dimensional.

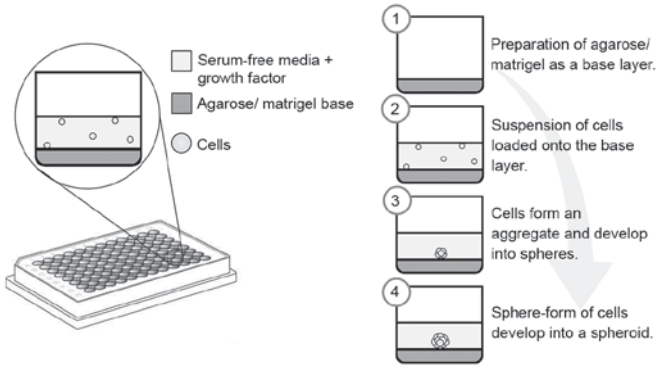

Figure 4. Formation of spheroids using either agarose or Matrigel as the base layer. Various concentrations of agarose or Matrigel may be applied as the base layer. The cells are suspended in serum-free medium with addition of growth factors such as basic fibroblast growth factor and epidermal growth factor. After four days, cells aggregate and form spheroids.

allows the antitumor effects of small molecule inhibitors and other chemotherapeutic agents, to be determined economically in a physiological environment. This may accelerate the drug screening process from laboratory to clinical trials. A preliminary study of a cancer tissue-originated spheroid (CTOS) demonstrated the use of 3D spheroid culture to form spheroids from human colorectal cancer, non-small cell lung cancer and urothelial tumors (63). CTOS retained the characteristics of the original tumors, a feature that may be utilized not only for drug screening, but also for future personalized therapy (64).

The terms 'sphere', 'sphere-forming' and 'spheroid' are also used in stem cell (SC) and cancer SC (CSC) research to refer to non-adherent cell growth in a serum-free medium which is typically supplemented with growth factors (65). The 3D spheroids are typically formed by the cells coming together to a compact ball or cluster of cells due to gravitational pull with a focus point formed by the meniscus of the agarose, as well as other factors depending on the type of method used to form the spheroid. The spheres in SC and CSC are also the same in the sense that their final appearance is essentially the same as a spheroid. However, the two principal differences between 3D spheroids and SC/CSC spheroids are the initial cell seeding number and the purpose of each assay. Three-dimensional spheroid culture generally uses at least 5,000 cells/well in a 6-well plate as an initial seeding number to begin non-adherent cell growth, whereas SC/CSC spheroid assays use ideally one cell/well in a 96-well plate as a seeding number to accurately assess self-renewal ability of putative SC or CSCs (66). In consideration of the difficulty of successful growth from a single cell for spheroid culture in SC/CSC research, a low clonal density of ≤20 cells/μl is considered appropriate for clonal growth in order to prevent cell aggregation or sphere fusion (66).

Similar to other tumor models, the 3D spheroid model offers divergent advantages and disadvantages. One of the disadvantages of the 3D spheroid model is the lack of tumor complexity, which can be overcome by the increase in the range and convolution of models applicable to researchers including co-culture system and patient-derived spheroids (67,68). Although we have the capability to recreate the tumor microenvironment architecture with a matrix of the 3D cell culture, variability in the matrix, particularly ones that are biologically derived, may yield non-reproducible results. However, the use of synthetic matrixes supplemented with growth factors may avert this issue (69).

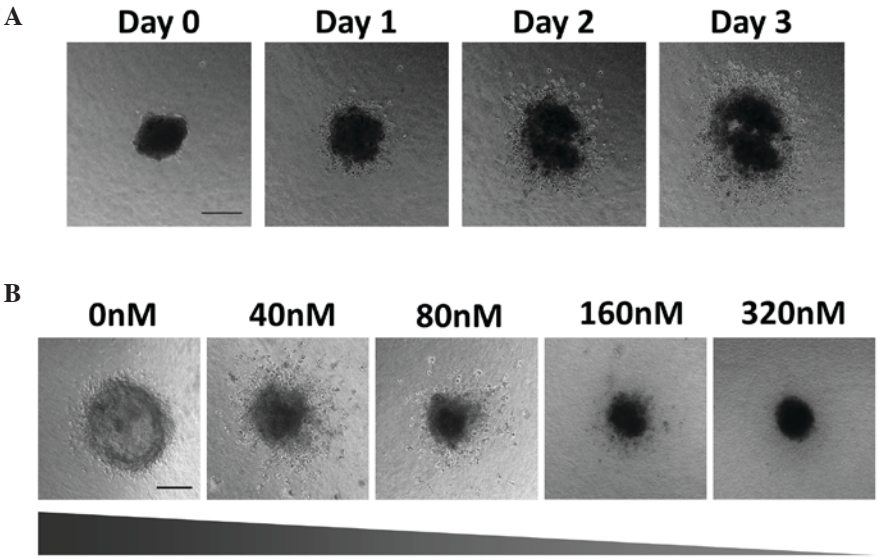

Figure 5. Growth and invasion of the HK1 spheroids into the collagen matrix over three days. (A) Phase contrast images of spheroid growth and invasion were taken every 24 h using the Nikon T_i Eclipse inverted fluorescence microscope; scale bar, 200 μm (B) Spheroids were treated with the chemotherapeutic drug Flavopiridol at the concentrations indicated. Phase contrast images taken every 24 h reveal that Flavopiridol induced a dose-dependent inhibition of spheroid growth and invasion (slope represents level of growth and invasion of the spheroids); scale bar, 200 μm.

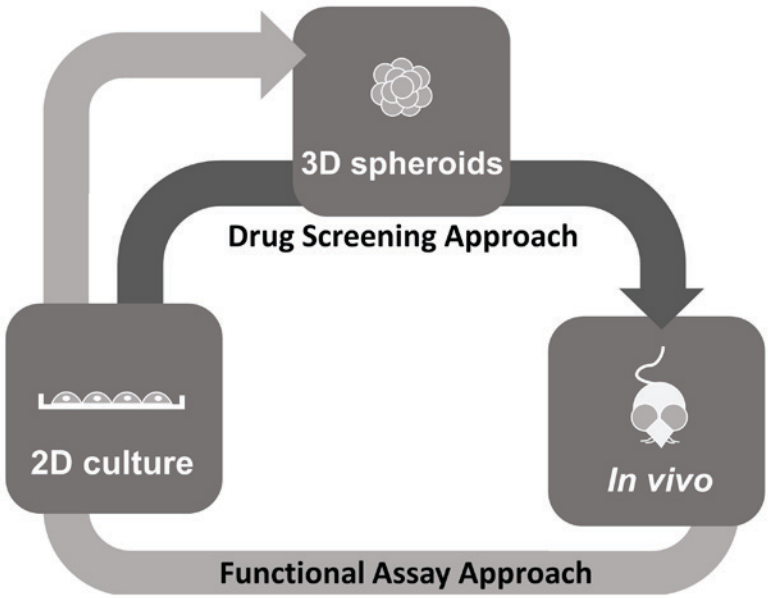

Figure 6. Proposed application of 3D spheroid models for therapeutic studies. Drug Screening Approach (dark grey arrow): The traditional 2D model is more economical and suitable for high-throughput screening compared with the 3D model. However, 2D models do not represent human cancer as *in vivo* models do. The transition from the 2D to the 3D model may allow for a relatively high throughput screening with the advantage of a more physiological model. The 3D model mimics the complex *in vivo* microenvironment, particularly with regard to gene expression, signaling pathways and drug sensitivity. The transition from the 3D model to animal models may also reduce the number of animals used for drug testing and may be more economical as animal models are costly and time-consuming. By using 3D models, more detailed data regarding drug suitability may be obtained prior to initiating *in vivo* studies with animal models. Functional Assay Approach (light grey arrow): *In vivo* tumor models may also be developed as *in vitro* 3D spheroid models for high-throughput functional assays. Cell lines can first be established and characterized in 2D from tumors obtained from the *in vivo* models such as the genetically engineered mouse models and xenografts and transitioned into 3D spheroids for functional studies. 2D, two-dimensional; 3D, three-dimensional.

5. 3D spheroid models for high-throughput drug screening

Inefficient drug delivery is one of the contributing factors to the mechanisms underlying drug resistance in human cancers (70). Despite possessing capacity for high-throughput drug screening, the 2D cell culture model cannot recapitulate the *in vivo* cellular architecture and microenvironment (71). In contrast, the 3D spheroid model used for high-throughput drug screening is able to more accurately predict *in vivo* drug efficacy and enhances the understanding, design and development of improved drug delivery systems (72). Compared with *in vivo* tumor growth, the size and uniformity of 3D spheroids are more consistent and adjustable, thus, making the model relatively more suitable for conducting drug screening (72). The

standardization of spheroid morphology, with regard to the uniformity of size and number, may be determined by the cell seeding density (72).

In order to improve drug screening using a 3D spheroid model, a previous study grew cells as multicellular spheroids rather than from spheroids from single-cell clones, as physiological human tumors are composed of multiple cell types, including epithelial cells, fibroblasts, mesenchymal stem cells, endothelial cells and immune cells comprising T, B and natural killer cells (37). The drug penetration into these multicellular spheroid aggregates enables investigation into the modulation of drug diffusion, sensitivity and resistance in cancers (37).

6. NPC 3D spheroids

A number of previous studies have developed improved NPC models based on spheroid cultures; these have increased in number due to novel culture methods emerging from the field of tissue engineering (Table I) (8,42,43,67,73-98). Formerly, the utilization of ultra-low attachment plates and hanging drop were the most common methods used to generate spheroids from NPC cells (74). Previous studies have used a liquid overlay culture method with improved medium cocktails to grow spheroids for NPC research (73,75). Several methods of 3D spheroid generation are presented and compared in Fig. 3.

As described, spheroids are established by the aggregation of cells creating a sphere-like structure, which interacts with the artificial ECM (12). The artificial ECM varies in composition depending on the method of spheroid culture. Some studies have utilized Matrigel, which served as a base for spheroid formation in the liquid overlay method (8,73,77,85,92). By contrast, the use of agarose-coated plates is more common in the liquid overlay method, with agarose being more economical compared with Matrigel (56). Furthermore, cell density and the cell medium cocktail also varies between studies (Table I). The processes of generating spheroids using agarose or Matrigel as the base layer are depicted in Fig. 4.

A number of previous studies have utilized spheroids generated from the C666-1 Epstein-Barr virus (EBV)-positive NPC cell line to study the effects of therapeutic agents (76-78). However, some studies have also used various EBV-negative NPC cell lines, including CNE1, CNE2, HONE-1, HONE-2, TW01, TW02, TW03, TW04, TW06, HK1 and SUNE-1 (references are presented in Table I). The application of spheroids generated from NPC xenografts is limited and primarily focused on the use of C15, a xenograft derived from a North African NPC patient, for drug evaluations (79,80). Spheroids generated from C666-1 cells were used to identify CSCs in EBV-positive NPC (81). C666-1 is a distinctive NPC cell line that preserves the native EBV genome (99), as EBV has been detected in nearly all non-keratinizing NPC cases (100). HK1 cells demonstrated the ability to form spheroids (Fig. 5). In addition, it was previously observed that HK1 contained a subset of cells termed side population (SP) cells, which were enriched with CSC-associated genes (101). A separate study identified certain CSC genes to be responsible for the formation of spheroids in HK1 (97). Together, this suggests an association between CSCs, SP cells and spheroids.

Other studies, which have utilized the spheroid model, previously identified that NPC CSCs also exhibited chemotherapy- and radiotherapy-resistance with self-renewal properties. Such NPC CSCs may potentially affect post-therapeutic cancer relapses (102,103); these studies may assist the development of new therapies for NPC. The 3D spheroid model may facilitate the identification of potential drugs that inhibit CSCs. Chan *et al* (76) identified that ICG-001, a specific CREB-binding protein (CBP)/β-catenin antagonist, blocked the CBP/β-catenin-mediated transcription of CSC-associated genes and synergistically reduced the formation of NPC spheroids when administered with cisplatin. In addition, spheroids may also be utilized in various assays such as cell differentiation assays (58), invasion assays (60), immunofluorescence staining and fluorescence-activated cell sorting analysis (74).

The similarity of 3D spheroids to *in vivo* tumors has enabled the study of growth, invasion and drug sensitivity of NPC HK1 spheroids towards the chemotherapeutic agent Flavopiridol in which HK1 cell spheroids were generated using the liquid overlay method (54,55). HK1 cells (~5,000) were seeded on hardened agar and incubated at 37˚C for three days. Once formed, the spheroids were embedded into a collagen matrix and medium was added to the solidified collagen matrix. It was observed that the HK1 spheroids grew and invaded the collagen matrix over three days (Fig. 5A). In a separate experiment, spheroids were treated with Flavopiridol at increasing concentrations for a period of three days from the day the spheroid was embedded (day 0). The results suggested that Flavopiridol inhibited spheroid growth and invasion in a dose-dependent manner (Fig. 5B). These cell line-derived spheroids have previously been used for selective (low-throughput) drug testing in NPC. The development of 3D spheroids for high-throughput drug screening for NPC may improve the methods of screening novel therapeutic agents for the treatment of this type of cancer.

7. Conclusion

The 3D spheroid model, which mimics the tumor architecture and microenvironment, offers a valuable approach to the study of NPC tumor biology in addition to providing a scalable system, which could allow the increase in quantity to meet the requirements for high-throughput drug screening. The 3D model enhances the selection of effective drug candidates and will allow for the prioritization of testing for drug candidates that seem the most effective prior to *in vivo* studies (Fig. 6). Additionally, high-throughput functional assays using these 3D models to assess the hallmarks of cancer, including cell viability, proliferation, apoptosis, cell cycle, migration and invasion may enhance the value of these models for the development of novel NPC therapies.

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