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Adaptable stirred-tank culture strategies for large scale production of multicellular spheroid-based tumor cell models

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ABSTRACT

Currently there is an effort toward the development of *in vitro* cancer models more predictive of clinical efficacy. The onset of advanced analytical tools and imaging technologies has increased the utilization of spheroids in the implementation of high throughput approaches in drug discovery. Agitation-based culture systems are commonly proposed as an alternative method for the production of tumor spheroids, despite the scarce experimental evidence found in the literature. In this study, we demonstrate the robustness and reliability of stirred-tank cultures for the scalable generation of 3D cancer models. We developed standardized protocols to a panel of tumor cell lines from different pathologies and attained efficient tumor cell aggregation by tuning hydrodynamic parameters. Large numbers of spheroids were obtained (typically 1000–1500 spheroids/mL) presenting features of native tumors, namely morphology, proliferation and hypoxia gradients, in a cell line-dependent mode. Heterotypic 3D cancer models, based on co-cultures of tumor cells and fibroblasts, were also established in the absence or presence of additional physical support from an alginate matrix, with maintenance of high cell viability. Altogether, we demonstrate that 3D tumor cell model production in stirred-tank culture systems is a robust and versatile approach, providing reproducible tools for drug screening and target verification in pre-clinical oncology research.

1. Introduction

The high attrition rates observed in cancer drug discovery (up to 95% of drugs tested in phase I clinical trials) have raised the awareness of the scientific and industrial communities toward the need for more advanced pre-clinical models. These models should be more reflective of the disease and consequently help to eliminate drug candidates that lack efficacy or safety at pre-clinical stages (Breslin and O'Driscoll, 2013; Ekert et al., 2014).

In this context, three dimensional (3D) *in vitro* cancer models can provide cell organization with higher physiological relevance when compared to typical two dimensional (2D) monolayers. Gene expression profiles, as well as phenotypic and functional characteristics of cells cultured in 3D are different from monolayer cultures and more closely resemble the features of the tissues of origin than their 2D counterparts (Lee et al., 2013; Guo et al., 2014; Ghosh et al., 2005; Kenny et al., 2007; Rohwer and Cramer, 2011; Friedrich et al., 2007). 2D cell cultures are easily established, monitored and characterized. Nonetheless, the spatial environment, cell morphology, polarity, receptor expression, oncogene expression and overall structural architecture are less reflective of the tumor environment than the 3D systems. 3D cell models enable tumor cells to establish and maintain cell–cell and cell-extracellular matrix (ECM) interactions, which are crucial mediators of cell signaling and modulators of response to therapeutic agents (Pickup et al., 2014; Howes et al., 2007). These interactions are essential for tumor cell differentiation, proliferation and survival *in vivo* (Seguin et al., 2015). Moreover, increased drug sensitivity due to higher cell surface exposure is expected in 2D cultures when compared to the *in vivo* conditions (Bechyne et al., 2012; Pampaloni et al., 2007). A 3D tumor model mimics the architecture of cancer cells in a tumor

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to a greater extent than 2D cell models and can therefore represent a more predictable model of drug sensitivity.

Multicellular spheroids were first established to investigate aspects of gastrulation movements in amphibian embryos (Holtfreter, 1944). Later, they attracted attention of the cancer research community after demonstration of similarity between the zonal morphological and functional distribution in aggregates of Chinese hamster V79 lung cells and histopathological structure of carcinomas from several patients (Sutherland et al., 1971; McCredie et al., 1971). The growth pattern of spheroids resembles the initial and avascular phase of solid tumors *in vivo*, not-yet-vascularized micrometastasis or intercapillary tumor microsites (Knuchel et al., 1988). These functional properties make spheroids a powerful model to investigate intercellular relationships and nutrient and oxygen diffusion in solid tumors (Kelm et al., 2003; Kumar et al., 2008). Despite the initial enthusiasm surrounding spheroids as cancer cell models, technical challenges have hindered their implementation in the drug development cascade (Hickman et al., 2014). To make this translation efficient, high levels of standardization are required, namely reproducible spheroid sizes and cell numbers. Additionally, the cell culture techniques must be amenable for scale up as high numbers of spheroids are required (Hirschhaeuser et al., 2010). Quantitative read-outs for 3D cultures were still limited which contributed for the choice of the research community to rely on 2D cultures as predominant tool (Hickman et al., 2014). Nevertheless, new analytical tools such as imaging technologies have been increasingly implemented into rapid and high throughput approaches for spheroid analysis (Gualda et al., 2014, 2015; Celli et al., 2014).

Diverse methods to produce spheroids have been developed over the years and currently there is no agreement on a single standard 3D model which could be used across the field. Force floating methods (low attachment plates or agarose underlayer) (Ivascu and Kubbies, 2006; Friedrich et al., 2009), hanging-drop method (Kelm et al., 2003; Amann et al., 2014; Fennema et al., 2013) or embedding in 3D matrices (Sodunke et al., 2007) are some examples of current strategies implemented to produce spheroids. Besides the static methodologies, dynamic approaches can also be used (Kelm et al., 2003; Lin and Chang, 2008). Agitation-based approaches to culture cells as spheroids include gyratory rotation techniques (such as gyratory shakers), rotary culture systems and stirred suspension culture systems (Hickman et al., 2014; Serra et al., 2012). In these dynamic approaches, hydrodynamic forces generated by the agitation are adjusted during cell culture to promote cell interactions and consequently cell aggregation (Alves et al., 1996). Increased shear, introduced by hydrodynamic culture environments, can regulate spheroid formation and control their size and shape through alterations in cell–cell collisions and adhesion binding kinetics at the cellular and molecular level, respectively (Kinney et al., 2011). In addition, the agitation-based cultures provide an efficient mass transport of nutrients and cell waste products to and from the spheroids, respectively (Serra et al., 2012). Large-scale stirred-tank culture systems present several features for the generation of an adequate culture environment for production of spheroids, namely reproducibility, scalability, adaptability and feeding for high throughput formats amenable to automation and drug screening (Hickman et al., 2014). Nevertheless, stirring rates must be tightly controlled to prevent physiological damage of the spheroids induced by the shear force (Serra et al., 2012).

For all these reasons, stirred-tank culture systems have been used for establishing 3D cultures due to their simplicity, low cost and efficient production of aggregates (Breslin and O'Driscoll, 2013; Serra et al., 2012; Benien and Swami, 2014). Nonetheless, despite the recognized potential for the production of tumor spheroids, these culture systems have not been used for the generation of a wide array of 3D tumor cell models for drug screening and target verification in oncology.

In this report we provide experimental evidence on the robustness, reliability and versatility of agitation-based culture systems for the large scale generation of 3D tumor cell models for a wide array of tumor cell lines. The robust character of stirred-tank culture methodologies herein described was demonstrated by the successful production of reproducibly sized spheroids from a panel of 9 different tumor cell lines from various pathologies, using the same cell inoculum. More than 10 independent biological replicates were performed for several of the tumor cell lines (MCF7, H1650, H157, HT29) with reproducible outcomes in spheroid concentration and size, highlighting the reliability of the proposed strategy. Moreover, the versatility of the stirred-tank culture methodologies was demonstrated by the application of different strategies for the establishment of mono- and co-culture 3D tumor models in the presence or absence of a 3D hydrogel. Altogether, we demonstrate that stirred-tank technology represents a fast and reproducible method, which may be used as 3D model source to feed high throughput screenings of new drug candidates.

2. Materials and methods

2.1. Cell lines and 2D cell culture

Human tumor cell lines derived from various cancer types (indicated in Table S1) were tested for their ability to grow as multicellular spheroids in stirred-tank culture systems.

Two ER^+ (MCF7 and BT474) and two ER^- (HCC1954 and HCC1806) breast cancer cell lines of human origin were selected. MCF7 and BT474 are among the most frequently used luminal-like cell lines and HCC1954 and HCC1806 are comprised within the subset of basal-like breast cancer cells. A549, H460, H1650 and H157 Non-Small Cell Lung Carcinoma (NSCLC) cell lines were selected for the development of lung cancer spheroids because they represent the major NSCLC subtypes: adenocarcinoma (A549, H1650), squamous (H157) and large-cell lung carcinomas (H460). HT29 was selected as colorectal carcinoma cell line.

H1650, H157, BT474, HCC1806, HCC1954 and HT29 cells were obtained from ATCC. Human Dermal Fibroblasts (hDFs) were obtained from Innoprot. MCF7 cells, transduced with the lentiviral vectors PGK-RFP and pCDH-CMV-MCS-EF1-Puro, were kindly provided by C. Brisken's laboratory. A549 and H460, modified to stably express TurboGFP using a lentiviral system (Sigma–Aldrich, SHC003V), were kindly supplied by W. Sommergruber, Boehringer Ingelheim. All above mentioned transformed cells were generated and provided under the scope of the Innovative Medicines Initiative Joint undertaking project, PREDECT (www.predect.eu).

2D static cultures (in t-flasks) were maintained at 37 °C in an incubator with humidified atmosphere containing 5% CO_2 and 21% of O_2. Tumor cells were sub-cultured twice a week at 1.5×10^4 cell/cm^2; hDFs were split once a week at a seeding density of 0.5×10^4 cell/cm^2. For each sub-culture, cells were trypsinized using 0.05% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; Invitrogen), for 3–5 min. Viable cell concentration was determined using trypan blue exclusion method (Section 2.5.1). All cell lines were routinely cultured in adherent and static conditions until establishment of the 3D cultures, using the culture media described in Table S1.

2.2. Generation of tumor cell spheroids in stirred-tank culture systems

Tumor cells were inoculated as single cell suspension into 125 mL or 500 mL wall-baffled spinner-vessels with straight blade