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In vitro 3-D multicellular models for cytotoxicity assay and drug screening

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ABSTRACT

Three-dimensional (3-D) cell culture models have been developed to improve drug screening and predictive efficacy. In this study, a high-throughput drug screening system with autofluorescent cells cultured in 3-D polyethylene terephthalate (PET) scaffolds mimicking *in vivo* microenvironment was developed. Using a modified microbioreactor platform designed specifically for 3-D cell cultures, three commonly used drugs, 5-fluorouracil (5-FU), gemcitabine, and sodium butyrate, were tested for their cytotoxicity on 3-D mouse embryonic stem (mES) cells and human colon cancer HT-29 cells, respectively. In general, 3-D cultures with multicellular structures exhibited similar expression in Ki-67 (a proliferation marker) and $p27^{kip1}$ (a quiescence marker) as compared to fresh tissues, and gave better predictive values of effective drug dosage *in vivo*. The 3-D multicellular mES and HT-29 cultures could be used as more reliable models for assessing dose-dependent drug responses, potentially reducing or partially replacing animal experiments, and thus should have applications in the early-stage drug discovery as well as toxicological evaluation of chemical compounds.

1. Introduction

Drug discovery and development is a complex, lengthy and expensive process, in which the actual costs of discovering and developing a drug lie between $800 million and $1.2 billion with development timelines spanning 8–12 years [1]. Considerable costs are incurred later in the pipeline due to high failure rates in full clinical development (Phases IIb and III). The major cause of attrition in the clinical trials is lack of efficacy, which often cannot be reliably predicted because available screening models are notoriously lacking of predictive power [2,3]. Historically, drug screening extensively relies on animal models as proxies for human beings in drug target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity). Although these animal models have the capacity to provide a wealth of useful information for drug screening, they are relatively expensive, low throughput and ethically challenging. Therefore, there is growing demand for *in vitro* assays for drug screening and improvements in these assays that could significantly reduce the cost and time-to-market of new therapies [2,4–6].

Currently, cell-based *in vitro* assays grow cells in two-dimensional (2-D) monolayer cultures as the predominant alternatives to animal models in primary drug screening [7]. However, 2-D cell-based assays cannot replicate *in vivo* counterparts and often cause discrepancy in predicting tissue specific responses [8–12]. Therefore, the current trend is to develop three-dimensional (3-D) cellular models that can better mimick *in vivo* tissues and give more reliable predictions on drug responses [13–17]. Various 3-D models for, such as, liver and skin have been developed [18–20]. However, it is challenging to monitor cell responses to drugs in 3-D cultures, which to date have been limited to end-point assay not suitable to high-throughput real-time dynamic drug pharmacokinetics studies [6]. Recently, we developed a label-free high-throughput fluorescence assay with cells expressing an enhanced green fluorescent protein (GFP) grown in a 3-D fibrous scaffold as an alternative *in vitro* method to study cytotoxicity and antineoplastic activity of drugs [12,21]. This 3-D fluorescent cell-based assay could be used to screen potential drugs, such as Chinese herbal medicines, for their cytotoxicity and anti-cancer activity [22,23].

In drug development, both efficacy and safety (cytotoxicity) of the drug must be screened. The goal of this study was to develop

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in-vitro tissue models for cytotoxicity assays with good predictive values on drug efficacy for screening cancer drugs. Mouse embryonic stem (mES) cells and colon cancer HT-29 cells were used to develop and demonstrate a working model for screening chemicals that are safe (with low or no cytotoxicity) and effective in treating colon cancer, the third most common cancer and the second leading cancer causing death, only next to lung cancer, in Western countries. Both the development of fetus and the existence of solid tumor are organized within a 3-D environment [24]. It is thus desirable to culture ES and colon cancer cells in 3-D scaffolds in developing a representative *in-vitro* tissue model for drug screening [25,26]. Three commonly used cancer drugs, 5-fluorouracil (5-FU), gemcitabine and sodium butyrate, were tested for their cytotoxicity effects on mES and human colon cancer cells. These chemicals were tested in 3 different models: 2-D monolayer model, 3-D low-density model, and 3-D high-density model with multicellular structures (tissue-like morphology). The results indicated that the 3-D multicellular model was more reliable in predicting tissue specific responses. This study demonstrated the use of an *in vitro* fluorescent HT-29 cell model to predict the efficacy and dosage of 5-FU for treating colon cancer.

2. Materials and methods

2.1. Chemicals

Both 5-fluorouracil (5-FU) and sodium butyrate were purchased from Sigma Chemical Company (St. Louis, MO), while gemcitabine was obtained from Eli Lilly Co. (Indianapolis, IN).

2.2. EGFP cell lines and media

Murine embryonic stem (mES) D3 cells (ATCC CRL-1934) and human colon cancer HT-29 cells (ATCC HTB-38) stably expressing enhanced green fluorescence protein (EGFP) were obtained by transfection with plasmid pEGFP-N3 (Clontech, Mountain View, CA) using Lipofectamine 2000 (Invitrogen, Grand Island, NY), following the method previously described [12,23]. After an initial 10-day screening using geneticin (G418, Gibco, Grand Island, NY), EGFP expressing colonies were isolated under a fluorescent microscope and then cells were subcultured in the absence of the selective pressure every 5 days for 20 passages. The stability of the transfected cell lines were verified (>97%) using FACS Calibur (BD Biosciences, San Jose, CA). EGFP expression was controlled with the human cytomegalovirus (CMV) promoter [27], a constitutive promoter giving stable and high expression of the fluorescence protein in proliferating mES and HT-29 cells [12,21].

Unless otherwise noted, ES D3 and ES-GFP cells were maintained on gelatin pre-coated T-flasks containing Dulbecco's modified Eagle's medium (DMEM, high glucose 4.5 g/L, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 μM monothioglycerol (Sigma-Aldrich) and 1000 U/mL leukemia inhibitory factor (LIF, Millipore, Billerica, MA). Both ES-GFP and its parental strain maintained a high stage-specific embryonic antigen (SSEA-1) expression level (>95%) in the aforementioned medium. HT-29 and HT-29-GFP were cultured in DMEM (high glucose 4.5 g/L, Gibco) supplemented with 10% FBS. These cultures were incubated in a humidified cell culture incubator at 37 °C with 5% CO_2.

2.3. Cytotoxicity assays in 2-D cultures

Each well on the 12-well plates was seeded with 50,000 cells, which were then exposed to chemicals one day after inoculation. Cell number in each well was quantified daily with Trypan Blue (Sigma-Aldrich) staining using a hemocytometer. IC_{50} was calculated as the concentration of chemicals resulting in 50% reduction in the viable cell density as compared to the control on day 3 for ES cells and day 6 for HT-29 cells.

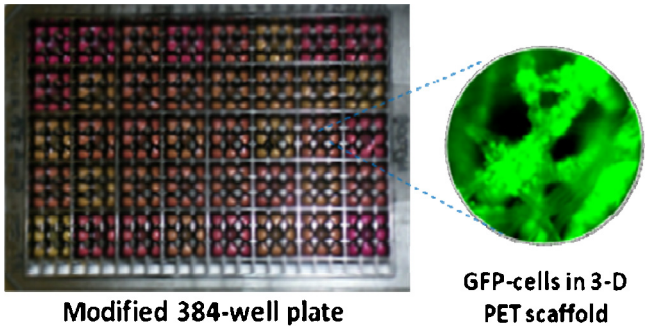

Fig. 1. Microbioreactors on modified 384-well plate used in the 3-D cytotoxicity assay. Each microbioreactor was made of 9 original wells on the plate with their walls removed. A PET matrix as cell scaffold was placed in the original center well for the 3-D culture.

2.4. Cytotoxicity assays in 3-D cell cultures

Unless otherwise noted, cells were cultured in nonwoven polyethylene terephthalate (PET) fibrous disks (fiber diameter: ~20 μm; disk diameter: 6.2 mm; disk thickness: 1 mm) in modified 96-well plates, each with 6 microbioreactors (working volume: 3 mL), each of which consisted of 9 original wells with their walls removed, as described in our previous studies [12,22]. Briefly, PET matrices were soaked in 10 μg/mL fibronectin for 12 h and then in the growth medium for another 12 h. After removing medium, each PET scaffold in a 96-well plate was seeded with 25,000 ES-GFP cells or 40,000HT-29-GFP cells in 25 μL medium. The 96-well plate was put into a CO_2-incubator for 6 h, allowing cells to attach to the fibrous matrix, and 180 μL of growth medium were then added to each well and incubated for another 24 h. Then, each cell-containing scaffold was transferred gently with a sterile tweezer to the modified 96-well plate for 3-D cytotoxicity studies. The cytotoxicity assay was also performed in a modified 384-well plate with 40 microbioreactors (Fig. 1), each with a 1-mL working volume [21]. Unless otherwise noted, 10,000 (ES-GFP) or 16,000 (HT-29-GFP) cells in 11 μL medium were seeded into each pretreated scaffold, which was then placed in the microbioreactor containing 1 mL medium. The multi-well plates were stacked onto an orbital shaker (Belly Button Shaker, Stovall, Greensboro, NC) at a rotational speed of 60 rpm in a CO_2-incubator. Chemicals including 5-FU, sodium butyrate and gemcitabine at different concentrations were added to the cultures 1 day (3-D low density models), 4 days (3-D high density ES-GFP model), or 8–10 days (3-D high density HT-29-GFP model) after inoculation. To measure the culture fluorescence, the plate was transferred to a fluorescent plate reader (GENios Pro, Tecan, Durham, NC) at 37 °C, with bottom reading as the measurement mode. All assays were conducted in triplicate or duplicate. Cell proliferation was estimated from fluorescence signal, which was proportional to the number of cells present in the 3-D PET matrix [12,21]. IC_{50} was calculated as the concentration of chemicals resulting in 50% reduction in the peak fluorescence signal as compared to the control.

2.5. Immunohistochemistry of cell cycle biomarkers

To evaluate the cell cycle progression of HT-29 cells in 3-D scaffolds and 2-D monolayer cultures, a cell proliferation marker Ki-67 and a quiescence marker $p27^{kip1}$ were studied using immunohistochemistry. Fresh human tonsil cells were used as control.