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Three-dimensional spheroid model using cancer and stromal cells for *in vitro* drug screening assays

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Abstract

One of the major drawbacks of using two dimensional cultures is that it does not reflect the physiology of modulators such as nutrients, oxygen, and metabolites of a solid tumor. Recently, there has been an explosion in studies related to developing 3D models to aid research in the fields of development, regenerative medicine and cancer. This interest is partly attributable to the interest in developing true to life models, inclusive of various cell types or extracellular components to reflect the physiological conditions of tumor microenvironment. In this short proof of concept study, we will demonstrate using natural products that a 3D system using stromal and cancer cells surpasses the sensitivity of 2D system.

Introduction

In classical cancer cell culture techniques, living cells coalesce, adhere, and grow in a single-layered lawn at the bottom of the plate in a two-dimensional (2D) fashion. Therefore, all cells are simultaneously attached to the plate and exposed to all conditions and any variables in the assay that may change. This classical 2D technique of growing and testing cells *in vitro* is efficient, however, it does not suitably replicate true cell growth *in vivo*. This creates a gap for researches to surmount when trying to replicate *in vitro* results in an *in vivo* setting. For example, if cancer cells are grown in the 2D technique, the same cells in an *in vivo* experiment may not react to the drugs the same way due to the way the cells grow and develop in the contrasting environments of the petri dish and animal body [1]. For this reason, three-dimensional (3D) culturing has been developed to bridge this gap that is currently left between classic 2D *in vitro* and *in vivo* studies [2]. 3D culturing allows for cancer cells to grow in spheres in the culture plate that more accurately represents cancer cell growth *in vivo*. Assays such as hanging-drop and sarcosphere/mammosphere methods have been developed to grow cancer cells in spheres [3,4], yet this leave very little opportunity for accurate visualization of the spheres in real time due to the lack of sphere adherence. Therefore, in order to visualize the spheres grown by these methods, the spheres must be transferred from low/non-adherent plates to adherent 2D plates, then stained in order for the spheres to be visualized [5]. This means the spheres cannot be used further in any following *in vivo* experiments. The inability to visualize spheres in real time and continue to use them in downstream assays is exacerbated when co-culturing is concerned. Generally, cancer growth is supported by stromal support cells recruited by the cancer to help the cancer cells continue to flourish [6]. By culturing mesenchymal stem cells (MSCs) with cancer cells, stromal cell cancer support is more accurately projected. However, without real time imaging, spheres of unknown size, density, and cell to cell ratio would be used, which compromises assay integrity [7]. Therefore, these methods of growing 3D spheres are not viable in a translational sense. For this reason, our team has developed a method of growing adherent 3D cellular structures. With the ability to grow adherent spheres, we can successfully visualize 3D sphere formation in real time while keeping the spheres alive and intact to transfer to *in vivo* assays. This means cancer/stromal spheres can be developed and regulated in a controlled *in vitro* setting that more accurately represents true tumor development, then transferred to an *in vivo* environment that will allow further sphere development. In this proof of concept paper, the methods described will prove the benefits of growing adherent 3D cell structures compared to classical 2D and 3D culture techniques using a breast cancer model (MDA-MB-231) and osteosarcoma model (OS) KHOS cells.

Methods

Cell culture

Frozen vials of characterized human MSCs (MSCs) at passage one were obtained from the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine and cultured in alpha-MEM (Invitrogen, Carlsbad, CA) supplemented with 17.5% of hMSCs-compatible Fetal Bovine Serum (Atlanta Biologicals, Flowery Branch, GA) and 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen). The serum-deprived MSCs were obtained as described previously [8]. Briefly, cells were cultured until they reached 80% confluency, washed three times with phosphate-buffered saline (PBS) and then cultured

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in media without fetal bovine serum. MDA-MB-231 and KHOS cell lines were obtained from ATCC, Manassas, VA. Both cancer cell lines were maintained in culture with Dulbecco's modified essential medium (Invitrogen), 10% FBS (Atlanta Biologicals, GA) and 100 U/ml penicillin, 100 µg/ml streptomycin.

DNA quantification assay (Cyquant)

After treatments, cells are washed with PBS twice and allowed to air dry. Plates are then frozen at -80°C for 20 minutes. Cells are then exposed to the lysis buffer/GR dye mix contained in the Cyquant kit (Thermo Fisher Scientific, Waltham, MA) for five minutes. After this step, the lysis/dye mix is transferred to a fresh plate to avoid any interference from cell debris. A DNA standard curve is used as a reference and the plate is then read using a fluorescence microplate reader (Synergy 4, BioTek, Winooski, VT) equipped with 480 nm excitation and 520 nm emission filters.

Expression, purification and conjugation of Elastin like Polypeptide (ELP)

Elastin like Polypeptide (ELP) was obtained from genetically modified *Escherichia coli* bacteria with gene for $(VPGVG)_{40}$ (V = valine, P = proline, G = glycine), by a suspension culture in terrific broth and purified by inverse phase transitional cycling, dialyzed against deionized water and lyophilized as previously described [9,10]. Chemical conjugation of ELP was done using polyethyleneimine (PEI, MW = 800 Da, Sigma, St. Louis, MO), using activation of ELP with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (Sigma) as described previously in [9,10].

ELP-PEI coating of plates

ELP-PEI coating was made accordingly to [9,10]. Briefly, ELP-PEI conjugate was coated atop a 96-well TCPS plate (Corning Costar, Corning, NY, USA) using 200 µL of 5 mol% ELP-PEI solution per well. The plate was kept in a dry incubator at 37°C for 2 days in order to evaporate the solvent and form the coating.

Natural products toxicity assay

Cancer cells were grown in 10% serum conditions while MSCs were grown in 17.5% serum or 0% serum (serum deprived conditions). Cells were then co-cultured into standard 96-well adhesive plates and allowed 24 hours for cells to adhere in 10% serum conditions. Next, the cells were exposed to various natural products for 24 hours. All natural products were prepared fresh from powered extracts dissolved in DMSO. Following natural product exposure, cells were washed with PBS and the DNA quantification assay (Cyquant) protocol was used per manufacturer protocol.

Live Imaging with fluorescent cell membrane staining

Live Imaging was done at the UMMC Non-Embryonic Stem Cell Core and School of Dentistry. KHOS and MDA-MB-231 cells were grown in 10% serum conditions until confluency was reached, cancer cells (KHOS or MDA-MB231 were stained with PKH67 (green fluorescence) and MSCs with PKH26 (red fluorescence) according to manufacturer's protocol (Sigma). Once stained, cells were transferred into regular 2D cell culture plates (Corning) or 3D ELP-PEI coated plates as described above in three different conditions: alone, co-cultured with MSCs, or co-cultured with MSCs exposed to serum deprived conditions (SD-MSCs). A time lapse protocol took pictures of selected areas of sphere congregation in each well every two hours for 60 hours using an EVOS Auto *fl* microscope (Thermo Fisher Scientific, Waltham, MA) or Olympus IX-81 microscope (Olympus, Center Valley, PA).

Results

2D vs 3D toxicity assays

To compare between 2D and 3D co-cultures, natural product toxicity assays were utilized. Cancer and stromal cells were grown in a timely manner in order to be harvested and re-plated at the same time into either 2D or 3D culture plates. Cells were allowed 24 hours to settle and begin sphere formation before being introduced to natural agents. On a panel of natural products, two most effective agents, turmeric and curcumin, were selected in a dose response curve type assay. After 24 hours of product exposure, cells were washed, stained, and analyzed using a DNA quantification analysis (Cyquant assay). In the 2D model, a 30.1 ± 7.6% decrease of cell proliferation is observed at a dose of 30 µg/mL turmeric (Table 1 and Figure 1). A further trend of decrease in proliferation is also observed when KHOS cells were co-cultured in presence of MSCs or SD-MSCs (40.3 ± 7.1% and 41.3 ± 7.2 % respectively). However, in the 3D model, a significant decrease of cell proliferation is observed compared to 2D model; First, KHOS cells proliferation significantly reduced at the dose of 30 µg/mL of turmeric (65.4 ± 7.2% vs. 30.1 ± 7.6%). Second, the co-culture of KHOS with MSCs or SD-MSCs display a significant stronger reduction of cell proliferation (85.1 ± 0.6% and 81.0 ± 2.0% respectively). The effect of curcumin, the refined product of turmeric, is moderated in 2D models but showed also more potent inhibitory effects in 3D model, especially in co-culture conditions (Table 2 and Figure 1).

Co-culture of stromal and cancer cells in 2D and 3D model development

In order to proceed with downstream sphere experimentation, we developed growth and imaging assays (as described above) to ensure heterogeneous sphere growth. First, a 2:1 cancer cell to stem cell ratio was determined in both the 2D and 3D models to be the best for sphere formation (data not shown). Spheres were visualized using normal 2D microscopy (Figure 2). Once this ratio was established, we repeated this assay with green (PKH67) and red (PKH26) fluorescence stained cells and visualized the spheres with 3D microscopy. This was used to confirm the sphere formation was heterogeneous which acted as a quality control for downstream experimentation on the uniformly formed spheres. As previously shown [11-13], serum deprived MSCs provide better stromal support and hence larger spheres both sarcospheres and mammospheres (Figures 3A and 3B).

Table 1. Inhibitory effects of turmeric on KHOS osteosarcoma cell proliferation in 2D and 3D cell culture models. Data are expressed as % of inhibition ± SE.

Conditions	2D	3D
KHOS	301 ± 7.6	65.4 ± 7.2
KHOS+MSCs	40.3 ± 7.1	85.1 ± 0.6
KHOS+SD-MSCs	41.3 ± 7.2	81.0 ± 2.0

Table 2. Inhibitory effects of curcumin on KHOS cells proliferation in 2D and 3D cell culture models. Data are expressed as % of inhibition ± SE.

Conditions	2D	3D
KHOS	20.2 ± 2.4	7.5 ± 3.4
KHOS+MSCs	18.1 ±1.6	21.9 ± 2.3
KHOS+SD-MSCs	16.7 ± 10.3	30.4 ± 4.5

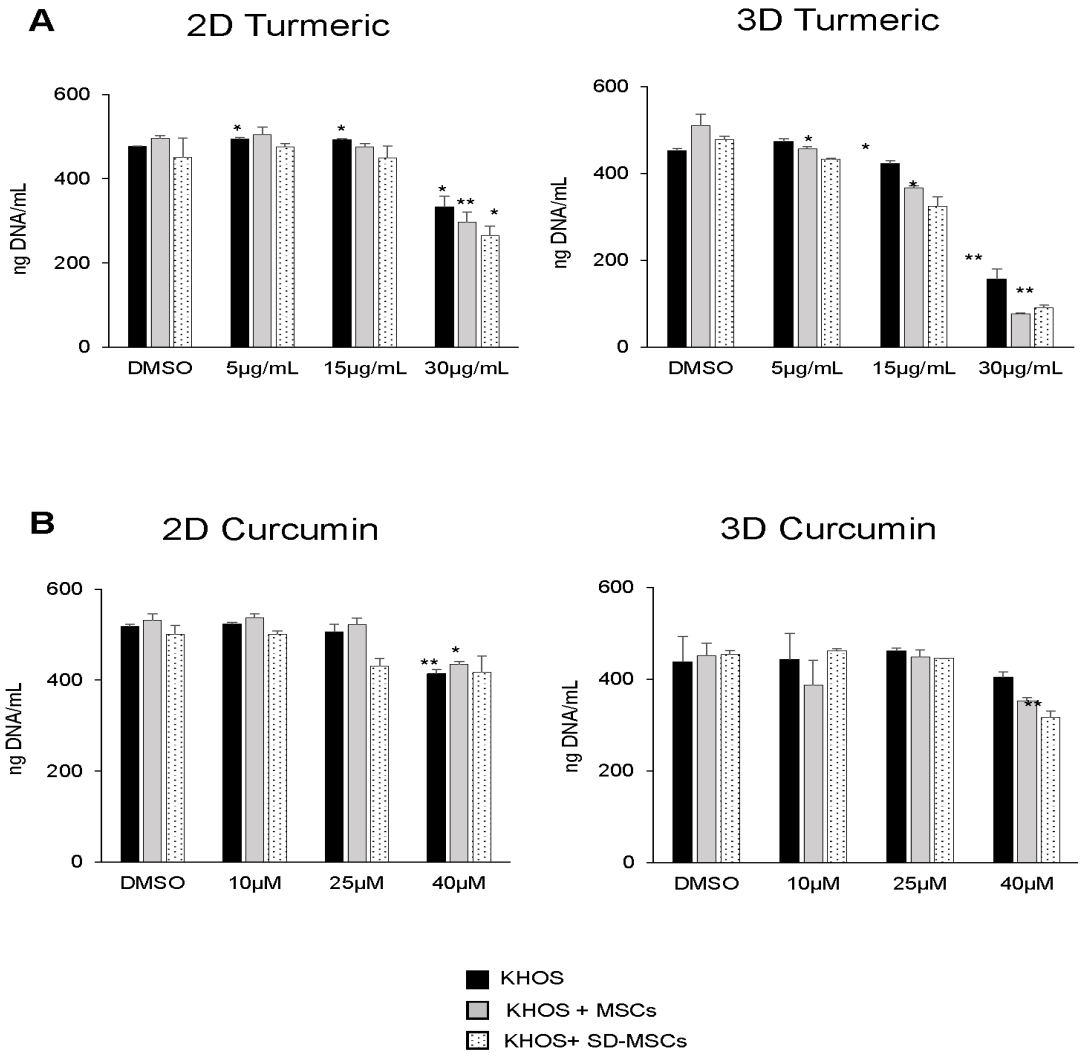

Figure 1. Graph representation of inhibitory effects of turmeric (**A**) and curcumin (**B**) on KHOs osteosarcoma cells proliferation in presence of normal mesenchymal stem cells (MSCs) or serum-deprived MSCs (SD-MSCs). *: $P<0.05$ and **: $P<0.01$.

Conclusion

Although proliferation was not seen in natural products, the lack of cell death in the 3D model also provides evidence as to the benefits of 3D model experimentation. Along with a more accurate tumor environment, we have proven that simple sphere visualization techniques and proliferation assays used in the 2D model can be easily adapted or directly converted for 3D utilization. Using both PKH26/67 staining and Cyquant protein proliferation analysis, our 2D and 3D data was easily compared and standardized with each other. This means researchers using the classical 2D models can transition to 3D *in vitro* models rather easily, which improves assay accuracy while not momentously shifting lab practices and protocols. The only main difference in 2D and 3D culture is 3D microscopy, which we used as quality control. Spheres can be visualized under normal 2D microscopy as long as fluorescence capability is adequate to ensure spheres are heterogeneous. Visualization notwithstanding, conversion from 2D to 3D cell culture provides an accessible and affordable *in vitro* model to more accurately assess the cancer/stroma environment. In comparing 2D and 3D models, it is clear the responses to curcumin and turmeric are varied suggesting the effect of conformation on toxicity assays.

A 2D model allows for consistent and even exposure to the cells leading to artificial effect of drug on cells, causing exaggerated drug efficacy. However, in the 3D model, sphere formation allows protection from toxic agents and allows the spheres to continue to proliferate under normal 2D dosages. The spheres natural resistance provides a more accurate response to toxic agents in *in vitro* environments, which leads to less error when trying to determine drug efficacy.

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Competing interests

The authors declare that they have no competing interests.

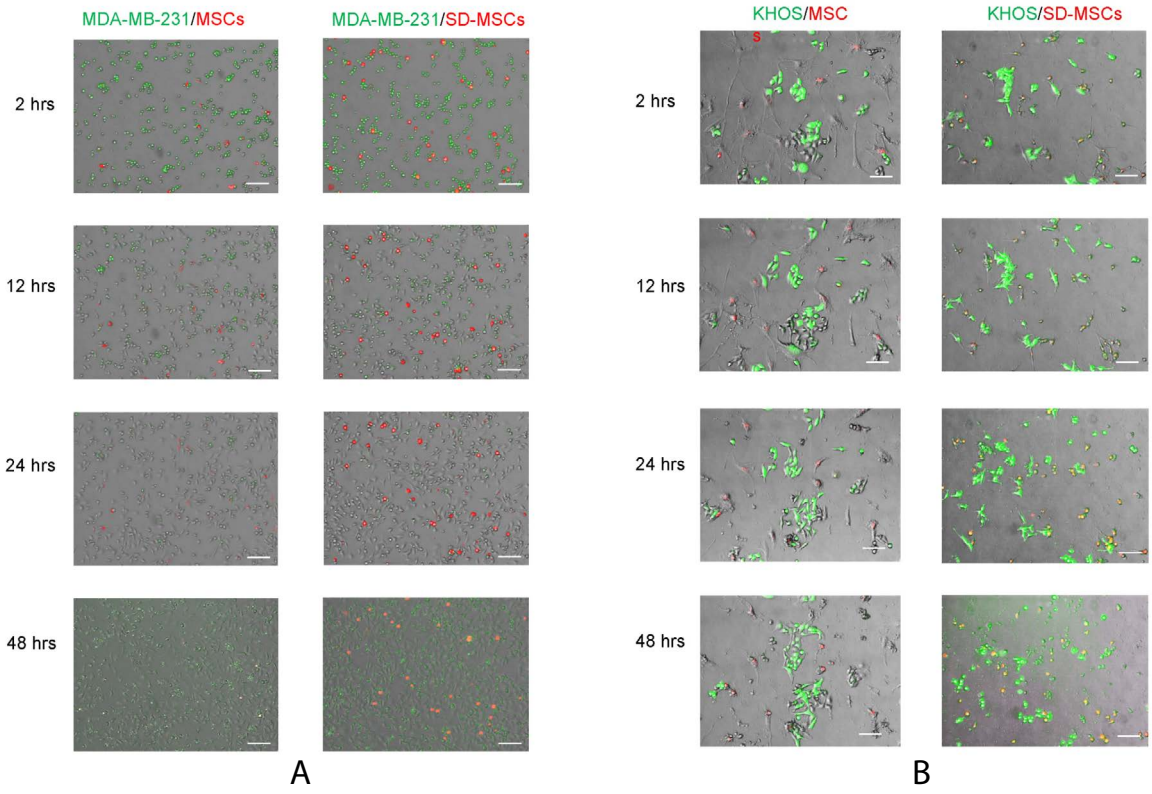

Figure 2. Time lapse of representative pictures of MDA-MB-231 breast cancer cells (**A**) or KHOS osteosarcoma cells (**B**) stained in green, co-cultured in classical 2D with normal mesenchymal stem cells (MSCs) or serum-deprived MSCs (SD-MSCs) stained in red. Pictures are a merged of transmitted light, GFP and Texas Red channels. Bar represents 200 µm.

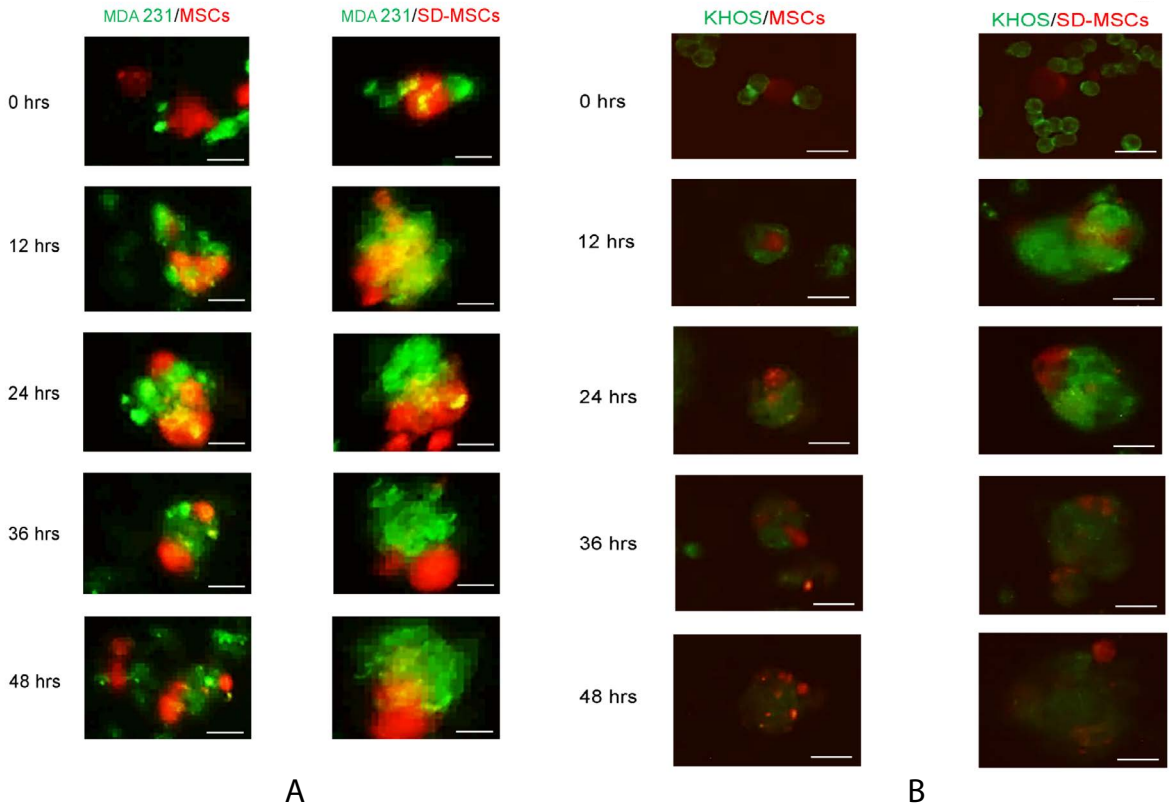

Figure 3. Time lapse of representative pictures of MDA-MB-231 breast cancer cells (**A**) or KHOS osteosarcoma cells (**B**) stained in green, co-cultured in 3D spheroids with normal mesenchymal stem cells (MSCs) or serum-deprived MSCs (SD-MSCs) stained in red. Pictures are a merged of transmitted light, GFP and Texas Red channels. Bar represents 100 µm.

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