Tumorsphere Passage for Breast Cancer Stem Cells

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Method Article

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Abstract

The tumorsphere assay allows one to assess whether a single cell harbors the potential to both initiate and maintain tumors in the absence of cellular interaction and adhesion. Stem cells are capable of unlimited self-renewal ability, and unique subsets of cancer cells that acquire stem cell properties have the theoretical ability to form de novo tumors when grown under low-attachment conditions within minimal growth factor supplementation. Primary stem cells and progenitor cells from the breast can be enriched within mammospheres, a concept that is analogous to neural stem cell enrichment in neurospheres. Tumorigenic efficiency of a subset of cancer cells can be determined based on number of spheres that emerge from single cells. On the contrary, cancer cells that lack stem cell properties have limited sphere-forming potential due to telomere loss and cellular senescence.

Reagents

ultra low-attachment 96-well plates \(Corning Costar) fetal bovine serum \(FBS) or fetal calf serum \(FCS) epidermal growth factor \(EGF) \(Invitrogen) basal fibroblast growth factor \(bFGF) \(Invitrogen) 50X B27 supplement \(Life Technologies) cancer cells \(primary or cell line) of choice trypsin-EDTA phenol red-free DMEM 40µm cell strainer cap filter \(BD Falcon) 0.4% Trypan Blue 1X phosphate-buffered saline \(PBS)

Equipment

Microscope \(fluorescent microscope if necessary) Flow cytometer with FACS capability \(for isolation of cellular subsets) Hemocytometer Centrifuge P1000 pipetter

Procedure

1. Preparation of sphere media: a. In serum-free and phenol red-free DMEM, add 20 ng/ml EGF, 20 ng/ml bFGF, and 1X B27 supplement. For example, to create 100 ml sphere media, add 2000 ng EGF, 2000 ng bFGF, and 2 ml of 50X B27 supplement. b. Alternatively, add 1% FBS \(v/v) into phenol red-free DMEM in the absence of EGF, bFGF, or B27 supplement. 2. Preparation of cellular subsets: a. Aspirate media from flask containing cancer cells, wash twice in 1X PBS, and trypsinize cells. b. Centrifuge cells at 300 x g at room temperature \(15-25°C) for 10 minutes. c. Decant supernatant and resuspend cells carefully in sphere media prepared from Step 1. d. Use 40µm cell strainer cap filter to obtain single-cell suspension. e. Isolate cellular subsets of interest via FACS or MACS \(if necessary). f. Determine cell viability via 0.4% Trypan Blue dye exclusion. g. Seed cell suspension at 1 cell per well in ultra low-attachment 96-well plate. 3. As a negative control, use non-tumorigenic breast cells, such as MCF-10 or MCF-12A. 4. Incubate ultra low-attachment 96-well plates under standard conditions at 37°C and 5% CO2. 5. Using microscope \(with fluorescent capability if necessary), assess wells daily for sphere formation. Allow plates to incubate for 10 days or more. 6. Calculate sphere-forming efficiency in the 96-well plate using the following equation: Tumorsphere efficiency \(%) = \(# of spheres) / \(# of wells seeded) x 100% 7. Serial

passaging of spheres a. Dissociate sphere mechanically using P1000 pipette or chemically using pre-warmed trypsin-EDTA. b. Centrifuge at 300 x g for 10 minutes at room temperature \(15°C-25°C). c. Prepare cells in sphere media created in Step 1. d. Seed 1 cell per well into a new ultra low-attachment 96-well plate. e. Assess sphere-forming efficiency in secondary, tertiary, and quaternary passages using formula from Step 6.

Timing

This assay will take approximately 3 hours for initial set-up. Total incubation time is approximately 10 days per passage. Timing varies depending on the doubling time of cancer cell population of interest.

Troubleshooting

Critical Steps Step 1: Be sure to use phenol red-free DMEM if fluorescence is required, as phenol red will interfere for excitation/emission and may create false positive results. Step 1: Note that serum-free media can sometimes lead to cellular death. If this occurs, it may be beneficial to supplement with 1% FBS or FCS to prevent death. Alternatively, one can use low-serum media in the absence of additional growth factor supplementation. Step 5: Be sure to allow enough time for spheres to form. This may take up to two weeks, based on the doubling time of different tissue types. Note that some cellular subsets may be more inherently quiescent and therefore require more time to initiate cell division and tumorsphere formation. Step 5: Note that morphology of spheres may vary depending on the cell line or primary tissue used. Some spheres may be more compact while others may be loose.

Anticipated Results

The most primitive and stem-like cancer cells are expected to have the highest sphere-forming efficiency. Spheres that arise from stem-like cancer cells may have intratumoral heterogeneity. This may be evident by most primitive cells located centrally and the most differentiated cells located peripherally. This assessment can be made by fluorescent microscopy if the putative stem-like cells are designated by a fluorescent reporter. Progenitors may have intermediate efficiency in forming spheres. Cancer cells that lack stem-like properties may initially form cell clusters, but these clusters will regress due to lack of self-renewal ability. The majority of the non-stem cells are expected to die by anoikis. Normal epithelial cells \(MCF-10 or MCF-12A) are not expected to form any spheres.

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