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Imaging and Analysis of 3D Tumor Spheroids Enriched for a Cancer Stem Cell Phenotype

FREDIKA M. ROBERTSON,^{1,2,3} MARCIA A. OGASAWARA,^{1,3} ZAIMING YE,¹ KHOI CHU,¹
ROSS PICKEI,^{1,3} BISLAT G. DEBEB,⁴ WENDY A. WOODWARD,^{2,4} WALTER N. HITTELMAN,^{1,3}
MASSIMO CRISTOFANILLI,⁵ and SANFORD H. BARSKY⁶

Tumors that display a highly metastatic phenotype contain subpopulations of cells that display characteristics similar to embryonic stem cells. These cells exhibit the ability to undergo self-renewal; slowly replicate to retain a nucleoside analog label, leading to their definition as “label-retaining cells”; express specific surface markers such as CD44⁺/CD24^{-low} and CD133; and can give rise to cells of different lineages (i.e., they exhibit multipotency). Based on these characteristics, as well as their demonstrated ability to give rise to tumors in vivo, these cells have been defined as tumor-initiating cells (TICs), tumor-propagating cells, or cancer stem cells (CSCs). These cells are highly resistant to chemotherapeutic agents and radiation and are believed to be responsible for the development of both primary tumors and metastatic lesions at sites distant from the primary tumor. Established cancer cell lines contain CSCs, which can be propagated in vitro using defined conditions, to form 3D tumor spheroids. Because the vast majority of studies to identify cancer-associated genes and therapeutic targets use adherent cells grown in 2 dimensions on a plastic substrate, the multicellular composition of these 3D tumor spheroids presents both challenges and opportunities for their imaging and characterization. The authors describe approaches to image and analyze the properties of CSCs within 3D tumor spheroids, which can serve as the basis for defining the gene and protein signatures of CSCs and to develop therapeutic strategies that will effectively target this critically important population of cells that may be responsible for tumor progression. (*Journal of Biomolecular Screening* 2010:820-829)

Key words: tumor spheroids, cancer stem cells, CyGELTM, thermo-reversible cell mountant, label-retaining cells, EdU, click chemistry, imaging, confocal microscopy, inflammatory breast cancer

INTRODUCTION

STUDIES OVER THE PAST 15 YEARS HAVE CONFIRMED the critical importance of small subpopulations of cells that persist within the bulk of human tumors that are capable of serving as tumor-initiating cells (TICs) or cancer stem cells (CSCs).¹⁻³ Based on techniques developed using neuronal precursors,^{4,5}

approaches have been developed that allow enrichment of CSCs from established cell lines as well as a variety of solid tumors. Using breast cancer as a primary example, studies have demonstrated that normal mammary epithelial cells as well as breast cancer cells can be propagated in vitro using serum-free medium containing growth factors and cultured low-adherence conditions.⁶ These culture conditions support the spontaneous formation of 3D tumor spheroids by cells from established breast cancer cell lines, tumor cells freshly isolated from biopsy samples of primary breast tumors, and tumor cells isolated from pleural effusion aspirates.⁶⁻¹⁰ Although culture conditions that allow enrichment of tumor cells with a CSC phenotype have been defined, there are significant challenges associated with imaging multicellular 3D tumor spheroids compared to imaging and analyzing cells cultured under conventional 2D adherent culture conditions attached to a plastic substrate. These challenges include approaches to stabilize the multicellular structures to image and characterize them and approaches to image and quantitate the property of self-renewal by cells within 3D tumor spheroids. An additional challenge is the analysis of surface markers and markers of multipotency that are expressed when cells are cultured as 3D tumor spheroids, compared to the traditional

¹The Department of Experimental Therapeutics.

²The Morgan Welch Inflammatory Breast Cancer Research Program.

³University of Texas Graduate School of Biomedical Sciences, Houston, TX, USA.

⁴The Department of Radiation Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA.

⁵Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA, USA.

⁶University of Nevada School of Medicine, Department of Pathology, Reno, NV, and Nevada Cancer Institute, Las Vegas, NV, USA.

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flow cytometric methods that require disaggregation of multicellular tumor spheroids to allow for analysis on a single-cell basis. Our goal is to describe approaches to image 3D tumor spheroid structures, allowing for characterization of specific traits of CSCs, including self-renewal as assessed by their clonogenic growth, their ability to retain a nucleoside analog label such as 5-ethynyl-2'-deoxyuridine (EdU), and their expression of specific surface markers such as CD44⁺/CD24⁻, as well as markers of bipotency, with detection of cells within 3D tumor spheroids that are of distinct lineages. We explore the major challenges of imaging and analyzing the activities of breast CSCs when cultured as 3D tumor spheroids and provide insight into potential solutions that allow multicellular tumor spheroids to be imaged and analyzed to further characterize signatures and therapeutic targets of CSCs.

MATERIALS AND METHODS

Cell lines and conditions

The MARY-X tumor spheroids were derived from the only xenograft model currently available that recapitulates inflammatory breast cancer in patients, as previously described, and are of the luminal B breast cancer subtype.¹¹⁻¹⁴ The SUM149 and SUM190 cell lines were developed from patients with inflammatory breast cancer and were generously provided by Dr. Stephen Ethier (Wayne State University, Detroit, MI).^{7,15,16} SUM149 cells are of the basal-like breast cancer subtype, and SUM190 cells are of the luminal B breast cancer subtype. MARY-X tumor spheroids and SUM149 and SUM190 inflammatory breast cancer cells were cultured at 37°C under 5% CO₂ in a humidified incubator. For low-adherence culture conditions, cells were plated into ultra-low attachment plates (Corning Life Sciences, Salt Lake City, UT) and maintained in mammary epithelial basal medium (MEBM[®]; LONZA, Hopkinton, MA) supplemented with 10 mL B27 (Invitrogen, Carlsbad, CA), 20 ng/mL epidermal growth factor (EGF; Invitrogen), 20 ng/mL basic fibroblast growth factor (bFGF; Invitrogen), 4 µg/mL heparin (Sigma-Aldrich, St. Louis, MO), and 2 mM L-glutamine to enrich for tumor spheroid formation.

Stabilization of 3D tumor spheroids

Tumor spheroids consisting of the SUM149 breast cancer cell line that contained stably transduced luciferase-IRES-green fluorescent protein (GFP), designated as SUM149-GFP, were stained with 5 µM of MitoSOX Red (Invitrogen) and with 20 µM of the nuclear stain DRAQ5[™] (Biostatus Ltd., Leicestershire, UK). Cells were incubated at 37°C for 10 min followed by washing twice with phosphate-buffered saline (PBS). CyGEL[™] (Biostatus Ltd.) was prepared for use as described by the manufacturer, cooled on ice, and made isotonic by addition of 40× PBS. CyGEL[™] was added to the 3D

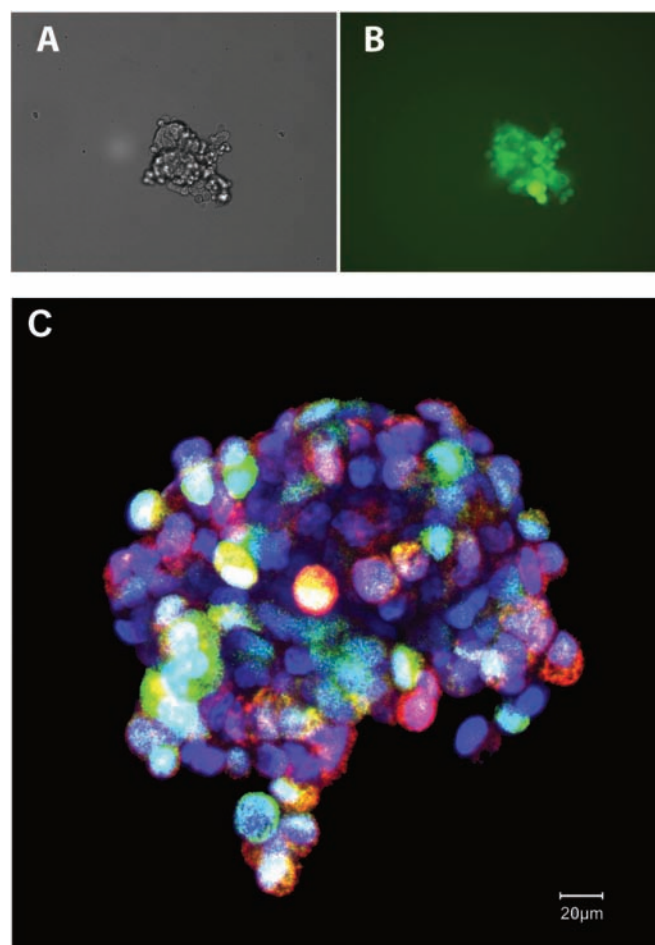


FIG. 1. Stabilization of 3D tumor spheroids. (A) Brightfield microscopy image of SUM149-GFP tumor spheroid in low-adherence culture conditions. (B) Fluorescence microscopy image of SUM149-GFP tumor spheroid in low-adherence culture conditions. (C) Confocal microscopy of SUM149-GFP tumor spheroid stained with DRAQ5[™] DNA dye and labeled with live-cell permeant MitoSOX dye, which is an indicator of superoxide anion production in living cells, stabilized using CyGEL[™] thermo-reversible mountant. CyGEL[™] allows higher resolution imaging of live 3D tumor spheroids cultured under low-adherence conditions without fixation or cytospin centrifugation.

SUM149-GFP tumor spheroids, and then cells were loaded onto cooled slides. Doubled-sided tape was used between the slide and coverslip to maintain the shape of the tumor spheroids. Imaging was performed using a confocal laser scanning system (LSM 510, Carl Zeiss, Peabody, MA; Fig. 1C).

Detection of CD44⁺/CD24^{-low} markers

To detect the presence of CD44 and CD24 surface markers, we fixed MARY-X tumor spheroids in cold methanol/acetone (1:1) for 20 min. Fixed tumor spheroids were then incubated

with 1% normal donkey serum diluted in PBS for 30 min to block nonspecific antibody binding. Cells were incubated with antihuman CD44 (clone 515, phycoerythrin [PE] conjugated; BD Pharmingen, San Jose, CA) and antihuman CD24 (clone eBioSN3, fluorescein isothiocyanate conjugated; eBioscience, San Diego, CA) for 45 min. The nuclei were counterstained with the DNA dye, Hoechst 33342 (5 $\mu\text{g/mL}$; Sigma-Aldrich). The stained cells were imaged on a Nikon fluorescent microscope with a Roper camera and MetaVue software (Universal Imaging Corporation, Downingtown, PA). A representative phase contrast image of a multicellular MARY-X tumor spheroid is shown in **Figure 2A**. Images of tumor spheroids stained with CD44/CD24/Hoechst 33342 are shown in **Figure 2B**.

Clonogenic growth as a surrogate marker of self-renewal activity of cells within 3D tumor spheroids

The ability of CSCs to exhibit self-renewal can be analyzed based on the ability of a single CSC to give rise to a clone of cells, defined as clonogenic growth, over a period of 14 to 21 days. To analyze clonogenic growth, we maintained single-cell suspensions of SUM149 breast cancer cells as described above. After 7, 14, and 21 days in culture, 0.5% thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich) was added to each well to visualize colonies formed by single cells, and samples were returned to the incubator for 1 h. Colonies derived from single cells were identified and quantitated using an Oxford-Optronix GelCount automated mammalian cell counting instrument (Sarasota, FL), and data were analyzed using GelCount software. As an example of the utility of clonogenic assays, studies were performed to assess the effects of the histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA), on clonogenic growth of breast cancer cells. Single-cell suspensions of SUM149 breast cancer cells were treated with either DMSO as the solvent control or with SAHA at concentrations of 0.25, 0.6, 1.2, 2.5, 5, and 10.0 μM , with each concentration run in triplicate. After 10 days in low-adherence culture conditions, tumor spheroids were labeled as described above, spheroid formation was measured by GelCount analysis of MTT staining, and data were analyzed using GelCount software. **Figure 3A** includes images of SUM149 colonies from the GelCount instrument. The effects of SAHA on clonogenic growth were quantitated, and the concentration at which SAHA inhibited 50% of colony formation, defined as the IC_{50} , was calculated using nonlinear regression analysis of average spheroid formation (**Fig. 3B**).

Clonogenicity assessed by analysis of anchorage-independent growth in soft agar

An alternative approach to the assessment of clonogenicity based on clonal efficiency is to analyze the ability of single-cell

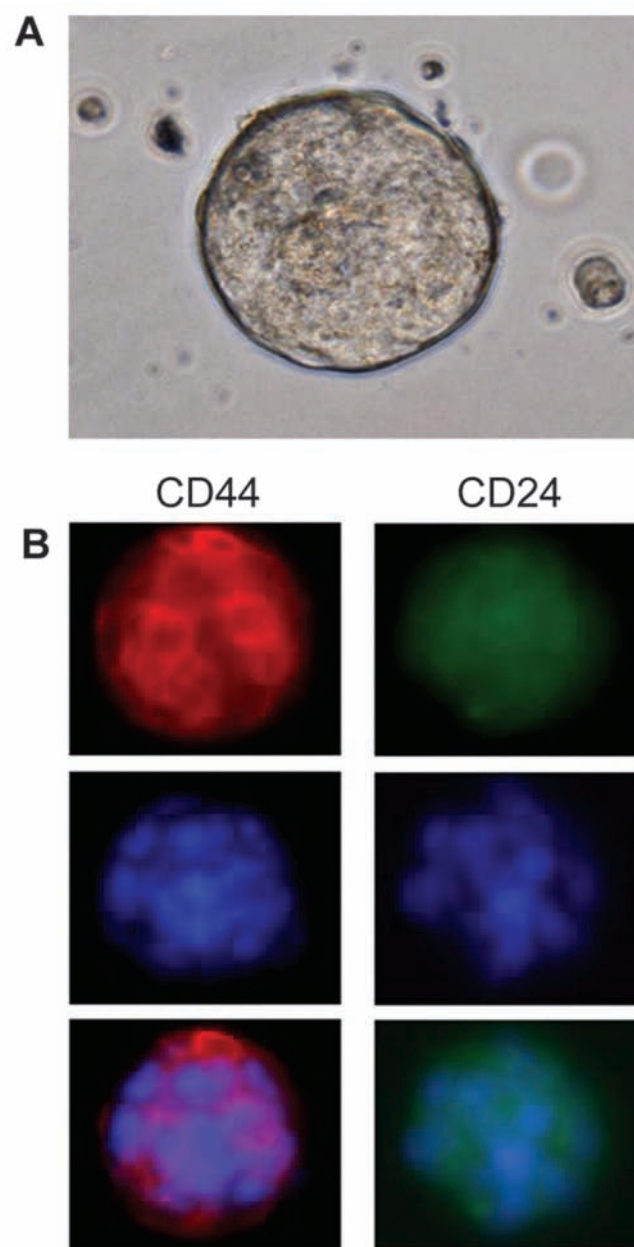


FIG. 2. Imaging of CD44/CD24 surface markers on 3D tumor spheroids. (**A, top panel**) In vitro, MARY-X appears as tumor spheroids, which appear under phase contrast microscopy as 3D multicellular structures composed of tightly aggregated tumor cells. (**B, left- and right-hand panels**) Immunofluorescence studies of the MARY-X tumor spheroids revealed strong expression of CD44 (top left-hand panel), but weak to absent expression of CD24 top (right-hand panel). The middle row in the left- and right-hand panels is Hoechst 33342 nuclear counterstain, and the bottom row in the left- and right-hand panels are composite images of CD44⁺/Hoechst 33342 DNA dye and CD24⁺/Hoechst 33342 DNA dye. Reprinted from *Am J Pathol* 2008;173:561-574, with permission from the American Society for Investigative Pathology.

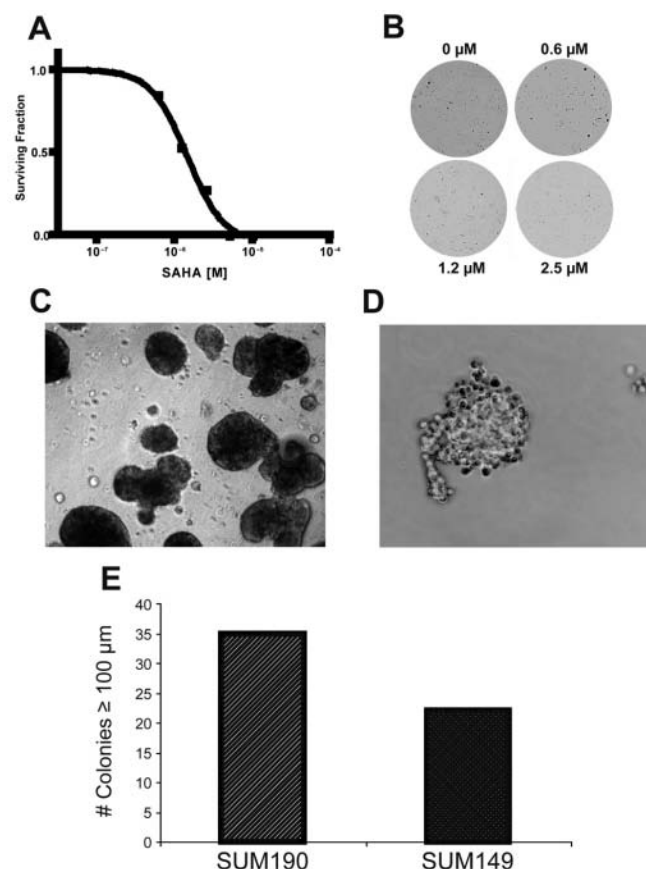


FIG. 3. Imaging clonogenicity of tumor spheroids as a measure of self-renewal capacity. (A) The dose-dependent effects of suberoylanilide hydroxamic acid (SAHA) on the number of spheroids formed by SUM149 are shown as a dose-response curve. The quantitation of the concentration of SAHA at which 50% of SUM149 tumor spheroid colonies were inhibited, defined as the IC₅₀, was calculated to be 1.2 μM. (B) Representative images of SUM149 colonies with self-renewal capability and visualization of inhibition of clonogenic growth of SUM149 by SAHA, using the GelCount instrument. (C, D) Representative light micrographs of anchorage-independent clones of SUM190 and SUM149 3D tumor spheroids, respectively, derived from single cells that exhibited self-renewal resulting in their growth in soft agar. (E) Quantitation of 3D anchorage-independent clones of SUM190 and SUM190 breast tumor cells that are equal to or greater than 100 microns in size.

suspensions of tumor cells to form colonies in a soft agar semisolid medium. Anchorage-independent growth of tumor cells disassociated from tumor spheroids was assessed by seeding cells into 6-well culture plates containing a base of 2 mL MEBM® medium containing 1% fetal bovine serum (FBS) and 0.5% agar in each well. Cells were disassociated from SUM149 and SUM190 cells and were plated into 6-well culture dishes containing the base of media, serum, and 0.5% agar using a cell concentration of 5×10^4 cells diluted in 2 mL of media containing

1% FBS and 0.35% agar. Plates were viewed twice weekly using phase contrast microscopy to determine time of visible colony growth. Studies were terminated at 14 to 21 days after seeding depending on the size of the colonies. Colonies were viewed and photographed, and the number and size of colonies were quantitated. Colonies developed from single cells were imaged using light microscopy, and a representative image of clones developed from SUM149 and SUM190 tumor spheroids is shown in **Figures 3C,D**. Quantitation of the number of colonies that grew in soft agar derived from single cells dissociated from SUM149 and SUM190 3D multicellular tumor spheroids that were 100 microns or greater in size is shown in **Figure 3E**.

Identification of label retaining cells within tumor spheroids

SUM149 tumor cells were cultured to approximately 75% confluence in adherent culture and then incubated with 5 μM EdU (Invitrogen) in F12 media (Invitrogen) supplemented with 10% FBS, 5 μg/mL insulin (Sigma-Aldrich), 1 μg/mL hydrocortisone (Sigma-Aldrich), and antibiotic/antimycotic (Invitrogen) for 4 h at 37°C. Cells were washed 4 times with Hank's balanced salt solution (HBSS), fresh media containing serum and supplements were added, and cells were cultured overnight. Cells were treated with trypsin to remove from culture plates, followed by treatment with soybean trypsin inhibitor (Sigma-Aldrich), and then maintained as tumor spheroids as described above. Cells were then filtered through a 40-micron filter and resuspended in MEBM® at a density of 25,000 cells/mL in ultra-low-adherence plates, and cells were allowed to grow for 7 or 14 days. Resultant tumor spheroids were placed onto slides using a cytospin cell centrifuge at 400 rpm for 3 min, and slides were air dried. Slides were rinsed with PBS, fixed in 4% paraformaldehyde diluted in PBS for 15 min at room temperature, and then washed in 3% BSA in PBS 3 times for 5 min. Cells were permeabilized using 0.5% Triton X-100 in PBS for 20 min at room temperature, followed by 3 consecutive washes in 3% BSA diluted in PBS. The "Click-iT" reaction cocktail (Invitrogen) was added to the cells using the manufacturer's instructions and incubated for 30 min at room temperature, followed by 3 washes in PBS. Cells were then incubated with the DNA dye TO-PRO-3 (Invitrogen) at a dilution of 1:300 in PBS for 15 min. Slides were then washed 3 times for 10 min each in PBS, mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and examined using fluorescence microscopy. Images were captured using a confocal laser scanning system (LSM 510, Carl Zeiss) at a 60× magnification. Z-stack images were collected through whole-tumor spheroids, and images were analyzed using Image J software and Adobe Photoshop. **Figure 4** shows representative images of SUM149 tumor spheroids labeled with EdU at time zero, 7 days and 14 days after labeling with EdU.

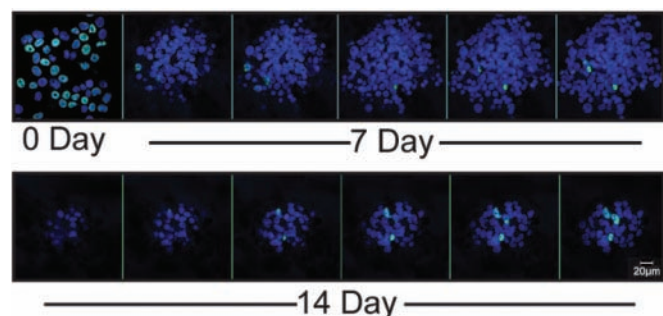


FIG. 4. Identification of label-retaining cells within 3D tumor spheroids. Confocal microscopy images of SUM149 cells within 3D tumor spheroid colabeled with the nucleoside analog EdU and TO-PRO-3 DNA dye at time zero. Over 7 to 14 days in low-adherence culture conditions, SUM149 tumor spheroids decreased in size, along with a decrease in the numbers of cells within the SUM149 tumor spheroid that retained both the EdU label and DNA dye. Quantitation of the cells that retained both EdU and DNA dye reveals that the “label-retaining cells” were 70% at time zero and 4% of the total population of cells within multicellular tumor spheroids at day 14.

Identification of bipotent CSCs within 3D tumor spheroids

SUM149 tumor spheroids were cultured in low-adherence culture as described above, maintained in culture for 7 to 14 days, and isolated and prepared for imaging by cytospin centrifugation onto slides at 300 rpm for 3 min; cells were rinsed with PBS and fixed in 4% paraformaldehyde diluted in PBS for 15 min. Slides were washed 3 times with PBS for 5 min each, and cells were then permeabilized using 0.1% Triton-X 100 in PBS for 5 min. Slides were then incubated in 5% normal goat serum diluted in PBS for 1 h to block nonspecific antibody binding. Slides were incubated with primary Cytokeratin (CK) 18 antibody (Clone DC10, Neomarkers, Fremont, CA) at a dilution of 1:500 and CK14 antibody (Clone AF64, Covance, Vienna, VA) at a dilution of 1:500 in PBS overnight at 4°C. Slides were washed 3 times with PBS and then incubated with either goat antimouse Alexa Fluor® 488 at a dilution of 1:400 for 60 min for detection of CK18 or with goat-antirabbit antibody conjugated to Alexa Fluor® 568 at a dilution of 1:400 for 60 min for detection of CK14. Slides were washed in PBS 3 times for 5 min each, incubated with TO-PRO-3 (Invitrogen) in PBS at a dilution of 1:300 for 15 min, washed 3 times for 10 min each in PBS, mounted in Vectashield mounting medium, and analyzed using a confocal laser-scanning microscopy system. Z-stack images were collected and projections were generated using Image J software.¹⁷ **Figure 5A-D** shows representative confocal microscopy images that allow visualization of tumor cells within the SUM149 tumor spheroid that are CK18⁺ and of luminal lineage, associated with Alexa Fluor® 488 green fluorescence (**Fig. 5A**); that are CK14⁺ and are of myoepithelial lineage, associated with Alexa Fluor® 568 red fluorescence (**Fig. 5B**); or

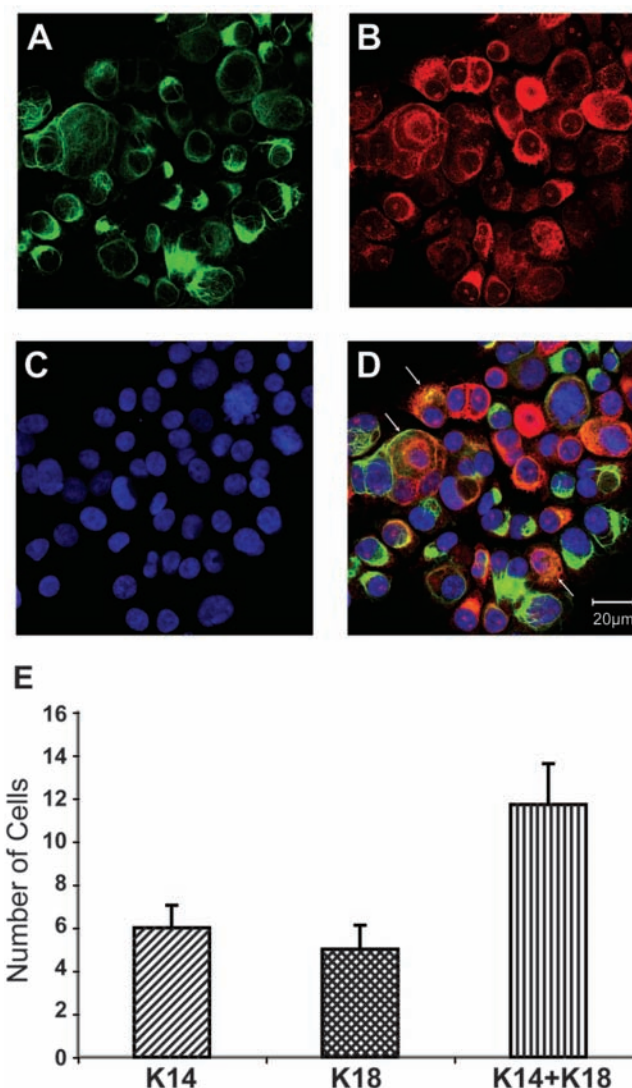


FIG. 5. Imaging bipotent cells within 3D tumor spheroids. Confocal microscopy images of (A) SUM149 tumor spheroids cultured under low-adherence conditions that stain with Cytokeratin 18 (green fluorescence associated with Alexa Fluor® 488), indicative of a differentiated luminal phenotype; (B) Cytokeratin 14 (red fluorescence associated with Alexa Fluor® 568), indicative of cells with a myoepithelial phenotype; (C) SUM149 tumor spheroid stained with TO-PRO-3 DNA dye; and (D, arrows) the merged image demonstrating the presence of cells that stain with both Cytokeratin 18 and 14, defined as bipotent cells. (E) Graphic representation of quantitation of cells within SUM149 tumor spheroids that are either CK18⁺ or CK14⁺ and those cells that are bipotent and are both CK18⁺/CK14⁺.

that are bipotent, associated with yellow/orange dual color (**Fig. 5D**). **Figure 5C** shows all cells stained with the nuclear dye To-Pro-3. **Figure 5E** shows the quantitation of cells that are CK18⁺, CK14⁺, or bipotent and are both CK18⁺/CK14⁺.

RESULTS AND DISCUSSION

Stabilizing 3D tumor spheroids for live-cell imaging

The first challenge in imaging tumor spheroids is to stabilize the 3D multicellular structures that will allow high-quality images to be captured and analyzed. Because the 3D tumor spheroids are in liquid media and are not adhered to a substrate, tumor spheroids are difficult to image when floating as free spheroids. **Figures 1A,B** shows brightfield and fluorescent images, respectively, of SUM149-GFP breast cancer cells cultured under low-adherence conditions, as an example of the low-resolution images of nonadhered tumor spheroids. To optimize the imaging of 3D tumor spheroids, we took advantage of the properties of a novel thermo-reversible cell mountant, CyGEL™, which is a liquid when chilled and rapidly reverts to a gel state above 21°C. CyGEL™ is inert, is optically clear, and has low autofluorescence, with a refractive index close to that of water. It is compatible with multiple fluorophores and can immobilize cells while retaining their viability, morphology, and protein expression, allowing for cells to be isolated, imaged, and further analyzed.¹⁸ Using CyGEL™, we obtained high-quality images of live cells within 3D tumor spheroids derived from established breast cancer cell lines. An example, shown in **Figure 1C**, is an image of a 3D tumor spheroid stabilized using CyGEL™. This method significantly enhances the ability to image a 3D tumor spheroid compared to the images obtained using either brightfield or fluorescence microscopy shown in **Figures 1A,B**. **Figure 1C** shows a SUM149-GFP tumor spheroid stained with 5 µM of MitoSOX Red and the nuclear stain DRAQ5™, demonstrating the utility of this approach to imaging live unfixed tumor spheroids to analyze such parameters as the production of superoxide anion using the MitoSOX Red indicator by cells within the 3D tumor spheroid. Further refinements of the techniques for immobilizing and imaging live 3D tumor spheroids using these approaches are under way. For longer term imaging of 3D spheroids, DRAQ5™ can be replaced with DNA dyes that do not alter turnover of DNA, such as Vybrant® DyeCycle™ Ruby Stain, a new dye that has an emission >670 nm for analysis of cell cycle in living cells (Invitrogen). The ability to immobilize 3D tumor spheroids using CyGEL™ provides the opportunity to perform live-cell imaging of 3D structures in real time. Using CyGEL™, 1 study was able to image and analyze live cells undergoing anoikis.¹⁹ Other uses for CyGEL™ include imaging of protozoan parasites such as *Leishmania major* and *Trypanosoma brucei*.²⁰ These studies demonstrated that the use of CyGEL™ proved to be a more rapid, easier, and more reproducible approach to live imaging of parasites compared to agarose, allowing for imaging of live parasites for up to 2 h with good viability. Another advantage of CyGEL™ is that by cooling CyGEL™, which results in its reversion to a liquid form, it can be used to recover either live cells or other organisms such as

parasites. CyGEL™ can also be “doped” with fluorescent agents such as DNA dyes, thus acting as a carrier for markers of viability. In addition, CyGEL™ can be used to stain organisms/cells of interest with organelle-specific probes, such as MitoSOX, shown in **Figure 1C**, that are not reliably delivered using agarose. Taken together with our studies demonstrating the utility of CyGEL™ for stabilization and imaging of live 3D tumor spheroids, these studies suggest that CyGEL™ allows a breadth of advanced microscopic techniques for live-cell imaging of both nonmammalian and mammalian 3D multicellular structures.

Identification of CD44⁺/CD24^{-low}-expressing cell subpopulations within tumor spheroids

CSCs have been shown to exist as relatively rare subpopulations within the bulk of tumor cells in a variety of different tumor types, including breast tumors, that can initiate tumors when injected into mice and appear to play a role in the development of distant metastasis.^{1,2,7,21,22} CSCs have been identified and quantitated based on the presence of specific surface markers, including CD44⁺, with a loss or lower level of expression of CD24, and epithelial-specific antigen.^{1,7} CD44 is a cell surface glycoprotein that functions as a hyaluronic acid receptor and is involved in cell-cell interactions. CD24 is also a glycoprotein but is expressed on cells with a more differentiated phenotype. A representative phase contrast image of a MARY-X tumor spheroid is shown in **Figure 2A**. An example of imaging of CD44⁺/CD24⁻ tumor spheroids is shown in **Figure 2B**, in which MARY-X tumor spheroids, which were developed from a patient with the most aggressive type of breast cancer, inflammatory breast cancer (IBC), were stained with PE-conjugated anti-CD44 antibodies and anti-CD24 antibodies conjugated with fluorescein isothiocyanate (FITC). Hoechst 33342 was used as the DNA counterstain to identify nuclei within cells of the tumor spheroid.

Numerous studies have now confirmed that cells expressing CD44⁺/CD24^{-low} are associated with a metastatic phenotype, suggesting that there is clinical relevance for characterization of CSCs expressing CD44⁺/CD24⁻ surface markers.^{21,22} Although CD44 appears to be a marker for less differentiated cells within solid tumors that can initiate tumors and have a role in development of metastatic lesions, interestingly, there is evidence that CD24⁺ tumor cells have phenotypic plasticity and can undergo alterations that result in their having increased expression of the CD44 surface marker.²³ Approaches to image CSCs in 3D are currently of great interest for their potential utility in identifying individual cancer patients who have tumors with very aggressive phenotypes, with the potential of developing individualized therapeutic strategies for each cancer patient based on definition of the characteristics of their tumor, including the expression of surface markers characteristic of CSCs. Although there certainly remains considerable

controversy about the importance and role of individual markers expressed by putative CSCs, there is no doubt that imaging approaches will remain critical to defining the role of these putative stem cells in tumor development and metastasis. Although flow cytometry remains the gold standard for characterization of CSCs that express specific cell surface markers associated with an aggressive phenotype, the disassociation procedures required to obtain single-cell suspensions for flow cytometric analysis result in loss of information about cell-cell interactions and the position of specific cells within 3D tumor spheroids.

Identification of cells capable of self-renewal

One well-characterized trait of CSCs is their ability to undergo self-renewal, which maintains the CSC population and, in some cases, can result in expansion of the pool of CSCs.⁷ Self-renewal can be determined based on analysis of clonogenicity of single cells disassociated from 3D tumor spheroids and based on analysis of growth of anchorage-independent clones in a semisolid soft agar medium. The first method to quantitate self-renewal of CSCs is based on analysis of the ability of cells to exhibit clonogenicity, which is defined as the ability of a single cell to form a multicellular colony. The resultant formation of colonies derived from a single cell can be imaged and counted using an instrument that functions as an optical counter, in this case, the Optronix GelCount instrument. **Figure 3B** is a light micrograph showing images of colonies in culture plates that had been seeded with single cells derived from SUM149 tumor spheroids and propagated for 14 days. The quantitation of colonies compared to the number of SUM149 tumor cells plated is used to calculate the colony-forming efficiency. As an example of the utility of this assay, this approach can be used to determine the effects of chemotherapeutic agents on self-renewal of CSCs, which can be calculated as the concentration of chemotherapeutic agent at which 50% of the clonogenicity is inhibited, defined as the IC_{50} .⁷ As an example, this assay was used to assess the effects of the histone deacetylase inhibitor, SAHA, on the clonogenicity of SUM149 tumor cells (**Fig. 3A**) and to calculate the IC_{50} of SAHA as 1.2 μ M.

Another assay that has been used to assess the ability of cells to exhibit self-renewal is the quantitation of cells seeded as single cells that grow as anchorage-independent colonies in a semisolid medium such as soft agar. Anchorage-independent growth is an assay that has been used as a gold standard to assess clonogenic growth over the past 3 decades.²⁴ **Figures 3C,D** shows light micrographs of colonies in soft agar formed by SUM190 and SUM149 breast cancer tumor cells. Quantitation of the number of colonies that arose from single-cell suspensions of SUM190 and SUM149 tumor spheroids when grown in soft agar as anchorage-independent clones is shown in **Figure 3E**. Previous studies have reported that when cultured

under 3D conditions, breast tumor cells exhibit specific morphologies, adhesion patterns, and nuclear organization that can be grouped within 4 primary classes, including round, mass, grape-like, and stellate, which is associated with the invasiveness of these tumor cells as well as their derivation from patients with metastatic disease.²⁵ The morphology of clones derived from SUM190 and SUM149 tumor spheroids, shown in **Figures 3C,D**, demonstrates that these breast cancer cells exhibit notable differences in their shape and size of colonies. The SUM190 cells exhibit tight cell-cell adhesion with disorganized nuclei, defined as having a “mass” morphology, and SUM149 cells from colonies can be classified as having a “grape-like” morphology, with disorganized nuclear and loose cell-cell adhesions. Although both SUM 190 and SUM149 cells were derived from patients with the most aggressive form of breast cancer, inflammatory breast cancer, they differ with respect to their invasiveness.²⁶

In addition to the utility of these methods to image and analyze cells capable of self-renewal, the use of the soft agar assay provides a means to expand specific populations of cells capable of exhibiting self-renewal isolated from primary tumors, pleural effusion aspirates, and tumor cells within bone marrow aspirates of patients or circulating tumor cells isolated from serum of patients bearing solid tumors. These methods provide the capability to expand populations of cells with self-renewal characteristics, which allows for their subsequent isolation and characterization of the genomic, proteomic, and microRNA signatures of these rare populations capable of self-renewal that are often resistant to conventional therapies and radiation and have been demonstrated to be involved in both development of primary tumors as well as metastatic disease.

Imaging and quantitation of label-retaining cells within 3D tumor spheroids

Similar to embryonic stem cells, CSCs have a very slow turnover time and therefore can take up and retain a DNA label, which has resulted in their definition as label-retaining cells.^{27,28} These slowly cycling cell populations have routinely been identified and quantitated *in vivo* based on analysis of the kinetics of the retention of nucleosides such as ³[H]-thymidine or the nucleoside analog bromodeoxyuridine (BrdU).²⁹ For *in vitro* studies, label-retaining cells have most commonly been detected and quantified using flow cytometry and/or confocal microscopy with antibody-based detection of the incorporation and retention of BrdU.³⁰⁻³² More recently, another nucleoside analog, 5-ethynyl-2'-deoxyuridine (EdU), has been shown to be useful to identify and quantitate cells that incorporate nucleoside analog into their DNA and retain it over long periods of time (>10 days) *in vitro*.^{33,34} EdU is incorporated into the DNA of cells in the S phase of the cell cycle and can be detected using “click” chemistry, which is a relatively new chemical system based on a copper catalyzed covalent chemical reaction

between an azide and an alkyne.^{35–38} This reaction has proven very useful in allowing the irreversible attachment of small molecular probes to a wide array of biomolecules for in vitro analysis of multiple parameters. For this protocol, the EdU solution contains the alkyne, and the dye of choice, such as Alexa Fluor® 488 dye, contains the azide. There are several advantages to using EdU labeling combined with click chemistry compared to using antibody-based detection of BrdU. To allow access of anti-BrdU antibodies into the nuclear DNA containing incorporated BrdU, extensive denaturation by acid, heat, and/or enzymatic treatment with DNase is required. The denaturation steps required for detection of BrdU incorporation can lead to significant distortions of cell morphology and are not readily amendable to imaging and analysis of label-retaining cells in multicellular 3D spheroids. In contrast, the small size of the dye azide allows for efficient detection of incorporated EdU with no denaturation requirement. The protocol from the manufacturer of the EdU Click-iT detection system (Invitrogen) uses a standard paraformaldehyde-based fixation and permeabilization with the detergent saponin, which allows the detection reagent to rapidly stain EdU incorporated into DNA without alterations in cell or nuclear morphology. This staining protocol is compatible with cell cycle dyes and can be multiplexed with antibodies against both surface markers as well as with intracellular markers. The detection of EdU using this approach allows assays to be multiplexed, with a choice of fluorescent probes that can be used for detection of label-retaining cells in combination with detection of multiple other surface and intracellular markers. Currently, EdU incorporation using click chemistry detection methods cannot be used for live-cell imaging, but there are now approaches using copper-free click chemistry reactions that have been shown to be useful for labeling live cells for in vivo imaging.^{39,40} With the continued improvements in the detection systems using click chemistry, it will likely be a very short time before we can detect multiple parameters, including label-retaining cells, among live cells within multicellular 3D spheroids. Our studies using EdU and click chemistry demonstrate that this approach provides a facile means to use confocal imaging to quantitate label-retaining cells within 3D SUM149 tumor spheroids over a period of 14 days. **Figure 4** shows representative images of SUM149 cells within a tumor spheroid labeled with EdU, showing the high percentage of EdU-stained cells (70%) at time zero, with a significant decrease in label-retaining cells at day 7 and day 14 under culture conditions that enrich for CSCs. At day 14 after incubation with the EdU label, 4% of cells within the SUM149 tumor spheroids retained EdU label. Approaches to identify and quantitate the cells within tumor spheroids that retain the EdU label can serve as the basis of assays to quantitatively evaluate the effectiveness of agents that may be able to target these slowly replicating label-retaining cells.

Imaging approaches to characterize multipotency of CSCs within 3D tumor spheroids

In addition to self-renewal, tumors that exhibit a highly metastatic phenotype also exhibit the ability to give rise to phenotypically diverse progeny that are within other lineages, consistent with the hierarchy of the tissue of origin.⁷ As an example of approaches to assess the multipotency of CSCs derived from established breast cancer cell lines, 3D SUM149 tumor spheroids were stained with antibodies directed against CK18, which identifies breast cancer cells within the luminal lineage, and with antibodies directed against CK14, which identifies breast cells within the myoepithelial cell lineage. As shown in **Figure 5**, within SUM149 tumor spheroids, there are cells that stained with only CK18 antibodies, detected as green fluorescence (**Fig. 5A**), and cells that stained with only CK14 antibodies, detected as red fluorescence (**Fig. 5B**). TO-PRO-3 was used as the DNA counterstain to identify nuclei (**Fig. 5C**). When images were merged (**Fig. 5D**), cells within the SUM149 tumor spheroid that stained with both CK14 and CK18 antibodies, which exhibit the characteristic of bipotency, were visible. **Figure 5E** is the graphic analysis of the number of cells within SUM149 spheroids that were CK18⁺, CK14⁺, or both CK18⁺/CK14⁺. These results are similar to previous studies that have reported that cells that exhibit a cancer stem cell phenotype are capable of bipotency.⁴¹

SUMMARY AND CONCLUSIONS

There is increasing evidence that tumor-initiating cells (i.e., cells with a CSC phenotype) may also be critical determinants of tumor latency and local recurrence and may be responsible for the development of metastasis at sites distant from the primary tumor. In addition, tumor cells with characteristics of CSCs exhibit plasticity and may be responsible for resistance to chemotherapy and/or radiation. The cancer stem cell hypothesis has significantly altered the approaches being used to define and characterize the specific population of cells that are believed to be the appropriate targets for the development of effective cancer therapies for multiple tumor types. It is now clear that the use of in vitro models of CSCs using low-adherence culture conditions that enrich for propagation of cells with CSC characteristics is critical to our understanding of the role and functions of CSCs. With this evolution in our understanding of the basis of tumor progression, there is a concomitant need for development of strategies to effectively image, isolate, characterize, and analyze tumor cells with the CSC phenotype in culture conditions that support formation of 3D tumor spheroids. The approaches described here may provide a basis for identification of cells with CSC characteristics within 3D tumor spheroids, allowing these relatively rare populations of cells to be analyzed. The methods described in the present study provide a means to analyze the ability of these specific

populations to retain label, undergo self-renewal and form anchorage-independent colonies derived from a single cell, and express specific surface characteristics, as well as identify the specific cells that retain their multipotency. Taken together with the increasing number of novel reagents such as the thermo-reversible CyGEL™ reagent that stabilizes live 3D tumor spheroids, allowing for high-resolution imaging of CSCs, and “click” chemistry combined with nucleoside analogs, allowing for identification of the cells capable of retaining label, investigators interested in analysis of the properties of CSCs now have an increased number of tools to add to their “technology tool kits” that will allow them to further characterize the role and functions of CSCs in tumor initiation and tumor metastasis using imaging and quantitative approaches that allow for analysis of 3D tumor spheroids.

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Address correspondence to:
Fredrika M. Robertson, Ph.D.
Department of Experimental Therapeutics
University of Texas M.D. Anderson Cancer Center
1515 Holcombe Blvd, Unit 422, Houston, TX 77030

E-mail: frobertson@mdanderson.org