Video Article

Utilization of the Soft Agar Colony Formation Assay to Identify Inhibitors of Tumorigenicity in Breast Cancer Cells

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Abstract

Given the inherent difficulties in investigating the mechanisms of tumor progression *in vivo*, cell-based assays such as the soft agar colony formation assay (hereafter called soft agar assay), which measures the ability of cells to proliferate in semi-solid matrices, remain a hallmark of cancer research. A key advantage of this technique over conventional 2D monolayer or 3D spheroid cell culture assays is the close mimicry of the 3D cellular environment to that seen *in vivo.* Importantly, the soft agar assay also provides an ideal tool to rigorously test the effects of novel compounds or treatment conditions on cell proliferation and migration. Additionally, this assay enables the quantitative assessment of cell transformation potential within the context of genetic perturbations. We recently identified peptidylarginine deiminase 2 (PADI2) as a potential breast cancer biomarker and therapeutic target. Here we highlight the utility of the soft agar assay for preclinical anti-cancer studies by testing the effects of the PADI inhibitor, BB-Cl-amidine (BB-CLA), on the tumorigenicity of human ductal carcinoma *in situ* (MCF10DCIS) cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/52727/

Introduction

Both non-transformed (normal) and transformed cells can readily proliferate in a 2D monolayer culture. This form of adherent cell growth is quite dissimilar from that which occurs *in vivo* where, in the absence of mitogenic stimulation, cells do not often rapidly divide within their microenvironment. The soft agar assay on the other hand is distinct from 2D culture systems because it quantifies tumorigenicity by measuring a cell's ability to proliferate and form colonies in suspension within a semi-solid agarose gel[1]. In this setting, non-transformed cells are unable to rapidly propagate in the absence of anchorage to the extracellular matrix (ECM) and undergo apoptosis, a process known as anoikis. In contrast, cells that have undergone malignant transformation lose their anchorage dependence due to activation of signaling pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt and Rac/Cdc42/PAK. Therefore, these cells are able to grow and form colonies within the semi-solid soft agar matrix[2].

A common use of the soft agar assay is to test whether specific compounds, such as PADI inhibitors, are able to suppress tumor growth *in vitro*. In general, colony count or colony sizes are quantitative read-outs from the assay that can be compared between control and treatment groups to assess differences in cellular tumorigenicity. Therefore, if one finds that colony formation is inversely correlated with increasing drug concentration, then a conclusion could be drawn that the drug is an effective inhibitor of tumorigenicity *in vitro*. On the other hand, if the drug does not affect colony formation, the drug is either not at the appropriate dosage or it is not an effective tumorigenic inhibitor. Aside from using a soft agar assay to test the anti-tumor effect of a drug, this assay can also be used to probe the relationship between a specific gene and tumorigenesis. For example, the effect of suppressing PADI2 expression on tumorigenicity can be addressed by PADI2-specific siRNA treatment.

PADIs are calcium-dependent enzymes that post-translationally modify proteins by converting positively charged arginine residues into neutrally charged citrulline in a process known as citrullination or deimination[3-5]. We have recently found that peptidylarginine deiminase 2 (PADI2) may function as a novel breast cancer biomarker and that PADI inhibitors represent candidate therapies for early stage breast cancers[6]. For example, we have previously demonstrated that a "pan-PADI" inhibitor, Cl-amidine, suppresses the proliferation of breast cancer cells using 2D monolayers and that the inhibitor suppressed the growth of 3D tumor spheroids[6]. In this report, we extend these studies, and highlight the utility of the soft agar assay, by testing the efficacy of a new PADI inhibitor, BB-CLA, in suppressing the growth of MCF10DCIS breast cancer colonies[7]. We note that we used MCF10DCIS cells for this experiment because they are oncogenic derivatives of non-transformed human MCF10A cells and because they contain high steady state levels of PADI2 protein[8]. We hypothesize that PADI2 enzymatic activity plays a key role in the tumorigenicity of this cell line and that BB-CLA-mediated inhibition of PADI2 activity will suppress cancer progression.

Protocol

1. Preparation of 3% 2-Hydroxyethyl Agarose

1. Into a clean, dry 100 ml glass bottle, add 0.9 g of 2-hydroxyethyl agarose (Agarose VII) followed by 30 ml of distilled water.
2. Microwave the mixture for 15 sec and gently swirl. Repeat this step at least three more times until the agarose powder fully dissolves.
3. Autoclave the solution-containing bottle for 15 min.
4. Allow the agarose solution to cool down to RT before further use. Store the solution at RT.

2. Preparation of the Bottom Layer: 0.6% Agarose Gel

1. Pre-warm several 5 ml and 10 ml pipettes in a 37 °C incubator to prevent the agarose from solidifying in the pipette when handling.
2. Partially loosen the bottle lid and microwave the pre-made 3% 2-hydroxyethyl agarose solution for 15 sec. Then, gently swirl the solution and microwave for another 15 sec. CAUTION: Be careful when swirling the agarose solution because the solution rises up when exposed to air and can spill over.
3. If there is residual solid gel in the bottle, microwave for a few more seconds.
4. Keep the bottle containing the agarose solution in a 45 °C water bath during the next steps to prevent the agarose solution from solidifying prematurely.
5. Warm MCF10DCIS media in a 37 °C water bath. Note: MCF10DCIS media consists of DMEM/F12, 5% horse serum, 5% penicillin streptomycin.
6. Transfer 3 ml of the 3% agarose solution using the pre-warmed pipettes into a sterile 50 ml conical tube.
7. Immediately add 12 ml of warm MCF10DCIS media and gently invert the conical tube to mix the agarose with the media. Try not to form any bubbles as it will interfere with the colony counting later.
8. Gently add 2 ml of this mixture into each well of a 6-well culture plate without forming any air bubbles.
9. Incubate the 6-well culture plate horizontally on a flat surface at 4 °C for 1 hr to allow the mixture to solidify.
10. After the mixture solidifies, place the plate into a 37 °C incubator for 30 min. The bottom layer is now ready for use.

3. Preparation of the Cell-containing Layer: 0.3% Agarose Gel

1. Trypsinize MCF10DCIS cells and dilute them to a cell concentration of 4 x 10^4 /ml.
2. Take 2 ml of the 3% agarose using pre-warmed pipettes and transfer into a sterile 50 ml conical tube.
3. Immediately add 8 ml of MCF10DCIS media to the conical tube and gently invert to mix the agarose with the media. Avoid forming any bubbles.
4. Take 2 ml of the MCF10DCIS cells (4 x 10^4 /ml) and treat with BB-CLA (0 µM (DMSO) or 1 µM).
5. In a 1:1 dilution, mix the cells with the 0.6% agarose.
6. Take 1 ml of the cell-agarose mixture and gently add onto the bottom layer of the 6-well culture plate (2 x 10^4 cells/ml).
7. Place the 6-well culture plate horizontally on a flat surface at 4 °C for at least 15 min to allow the top layer to solidify.
8. After the mixture solidifies, place the plate into a 37 °C incubator for a week before adding the feeding layer.

4. Preparation of the Feeder Layer: 0.3% Agarose Gel

1. Microwave the pre-made 3% 2-Hydroxyethyl agarose solution for 15 sec. gently swirl the solution and microwave for another 15 sec.
2. Equilibrate the agarose solution bottle in a 45 °C water bath.
3. Warm the MCF10DCIS media in a 37 °C water bath.
4. Mix 1 ml of 3% agarose solution with 9 ml of warm MCF10DCIS media into a 50 ml conical tube and gently invert to mix the agarose with the media. Avoid forming air bubbles.
5. Treat the mixture with BB-CLA (0 µM (DMSO) or 1 µM).
6. Gently add 1 ml of this mixture (without forming bubbles) into each well of the 6-well culture plate containing the bottom and soft layers.
7. Place the 6-well culture plate horizontally on a flat surface at 4 °C for at least 15 min to allow the mixture to solidify.
8. After the feeder layer solidifies, place the plate into a 37 °C incubator.
9. Repeat this feeding procedure weekly by overlaying 1 ml of 0.3% agarose/medium/treatment solution onto the existing feeder layer to replenish the cells with new media until colony formation is observed. Note: Agar in the soft and feeder layers is very soft and, therefore, the added nutrients from the feeder layer will readily diffuse into the cell-containing layer to reach the cells.

5. Data Collection

1. After 2.5 weeks of cell growth in the soft agar, count the number of colonies in each well using a light microscope. To facilitate quantification, print a grid onto a transparency and attach the grid to the 6-well plate to help locate where the cells are during counting. Since colony size (as quantified by the diameter of each colony) will vary, predefine a reference colony size to determine which colonies will be scored. For example, include colony sizes of 70 µm or larger in the data analysis.
2. Store the samples at 4 °C to prevent further colony formation and for future counting. Seal the 6-well culture plate with Parafilm to prevent the gels from drying out.

Representative Results

The soft agar colony formation assay can be used for a broad range of applications documenting the tumorigenicity of cancer cells. A major advantage of this technique is that the semi-solid matrix selectively favors the growth of cells that can proliferate in an anchorage-independent manner. This trait is mainly exhibited by cancer cells but not by normal cells. We primarily use this technique to test the efficacy of tumor growth inhibition by drugs and to test for the effect of overexpression or depletion of our genes of interest, including PADI genes, on the tumorigenicity of breast cancer cells. Here, we assessed the effect of BB-CLA on tumorigenic inhibition of PADI2-overexpressing MCF10DCIS cells **(Figure 1 and 2**).

The results demonstrate that BB-CLA significantly inhibits the formation of MCF10DCIS cell-derived colonies. **Figure 3** shows that, in the presence of a PADI inhibitor, there was a reduction in both colony formation and colony size when compared to the DMSO control. [Note: BB-CLA was dissolved in DMSO and thus, DMSO was used as a control.] The size of colonies for BB-CLA treated MCF10DCIS cells were predominantly within the range of 20 to 100 µm while the size of colonies for the DMSO control exhibited a greater range of 70 to 150 µm after 2.5 weeks of growth.

Colonies larger than 70 µm were counted and analyzed (**Figure 4**). There was an average of 3,536 colonies in the DMSO control whereas only 1,967 colonies were seen in the BB-CLA treated group after 2.5 weeks of soft agar culture. This represents a 44% decrease in the average colony formation in the presence of 1 µM BB-CLA, indicating a significant tumorigenic inhibition of breast cancer cells (MCF10DCIS cells) by the PADI inhibitor.

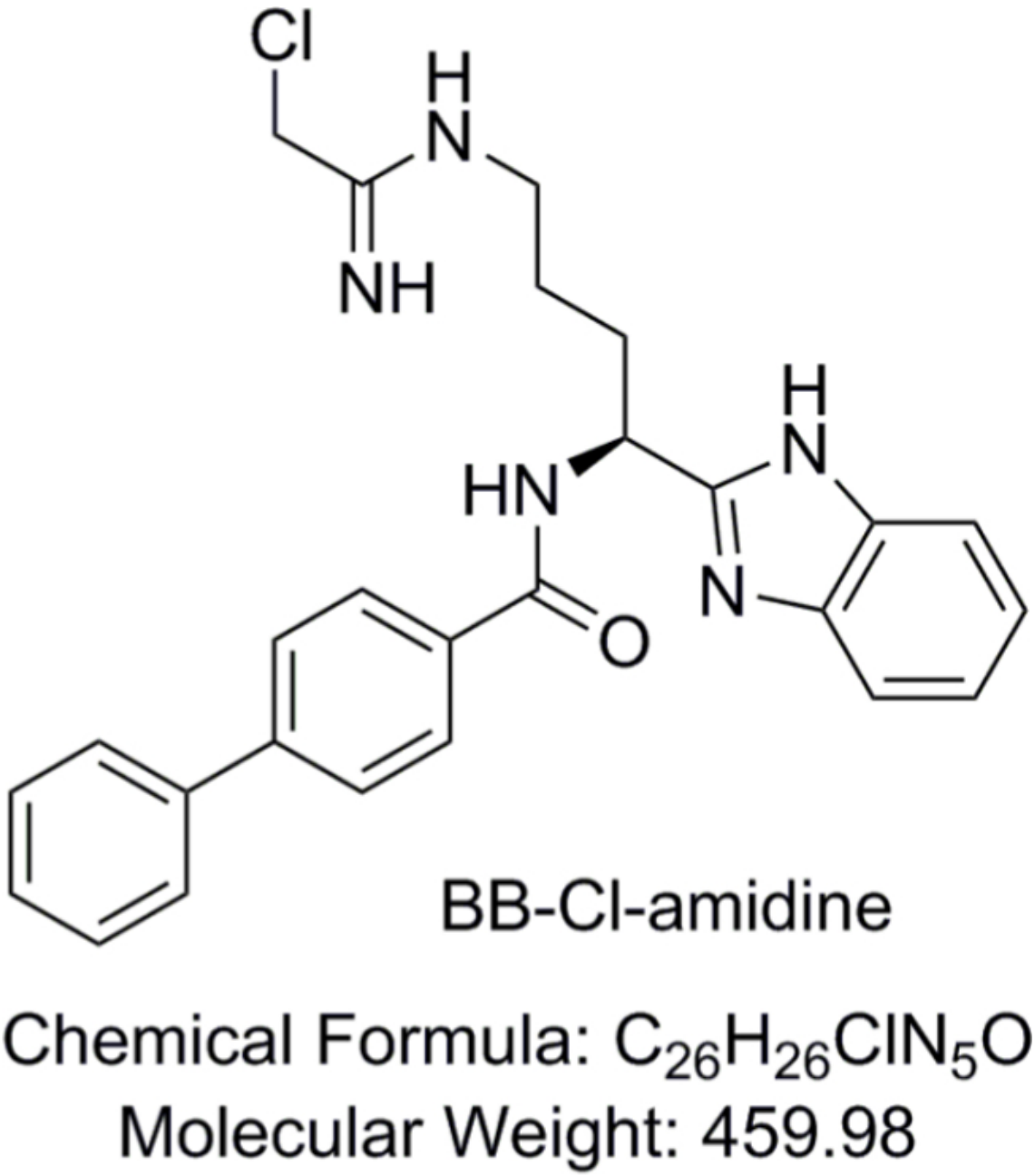

Figure 1. Chemical Structure of BB-CLA.

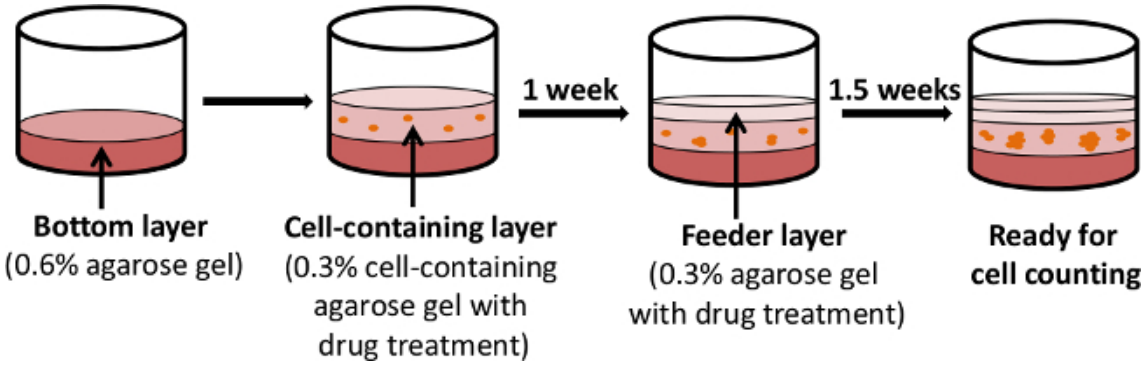

Figure 2. Schematic Overview of the Protocol for the Soft Agar Colony Formation Assay. Each well of the 6-well culture plates was first coated with 0.6% agarose gel (bottom layer). A 0.3% agarose gel mixture containing the MCF10DCIS cells and either the BB-CLA inhibitor (1 µM) or DMSO (control) was then layered on top of the 0.6% gel. Once a week, the 0.3% agarose gel mixture (containing BB-CLA) was added on top of the soft layer. After 2 to 4 weeks, colony formation was observed and counted for data analysis. Please click here to view a larger version of this figure.

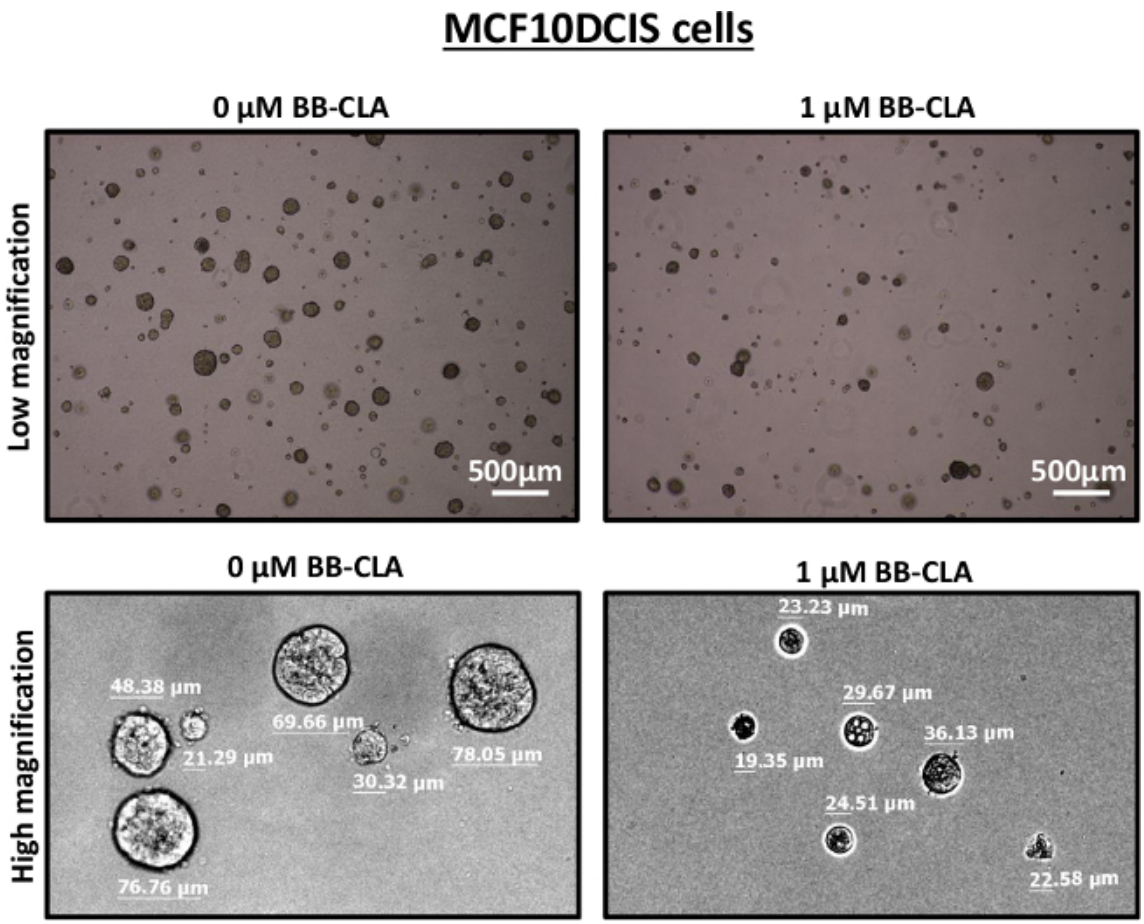

Figure 3. Images of Colony Formation in BB-CLA-treated MCF10DCIS Cells. MCF10DCIS cells were grown in soft agar in the presence (1 µM BB-CLA) or absence of BB-CLA (0 µM DMSO). After 2.5 weeks, colonies were imaged using an inverted microscope for low magnification images and a light microscope for high magnification images. Please click here to view a larger version of this figure.

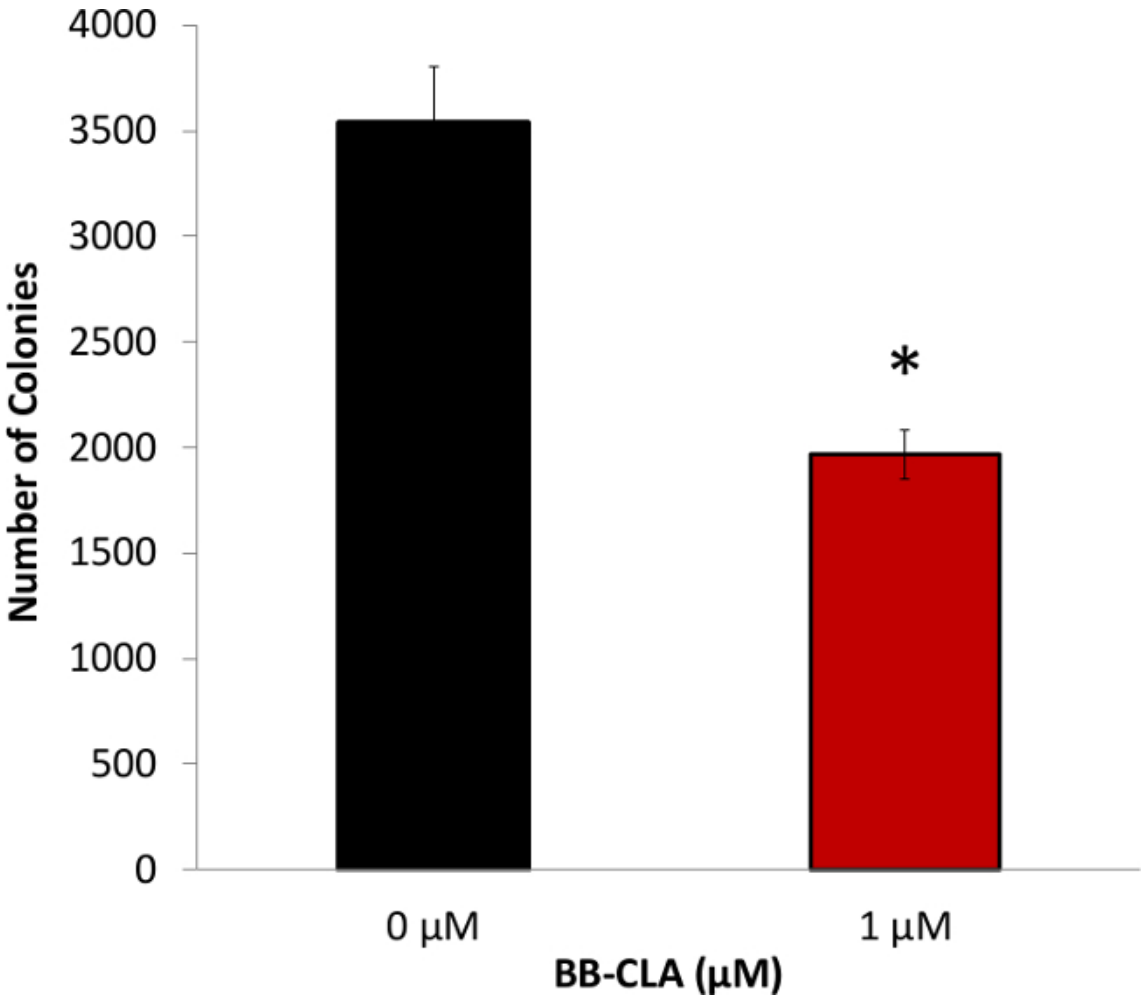

Figure 4. Quantification of MCF10DCIS Colony Number after BB-CLA Treatment. MCF10DCIS cells were grown in soft agar at different concentrations of BB-CLA (0 µM (DMSO), or 1 µM BB-CLA). After 2.5 weeks, individual colonies larger than 70 µm were counted. Experiments were repeated 4 times (n = 4). The statistical significance of total cell count difference between the control and treatment samples was determined by two-sample Student's t-test (*p < 0.005).

Discussion

The rate of colony formation in soft agar varies depending on the cell type[9]. Therefore, the number of cells to start with should be optimized and adjusted accordingly. A suggested starting range is between 5 x 10^2 to 1 x 10^4 cells per well using a 6-well plate. In addition, colony size varies depending on the growth rate of each cell. Therefore, a predefined a cut-off for colony size is needed to annotate individual colonies for downstream quantitative analyses. Here, colonies larger than 70 µm were quantified to avoid inclusion of non-proliferating cells derived from the initial plating.

For optimal growth, when making the 3D agarose gels, it is advisable to use the same cell culture medium normally used for 2D cell cultures. This is because changing medium often times alters the cells growth rate, thus necessitating additional passaging to allow the cells to adapt to the new medium. For example, MCF10DCIS cells can be grown optimally in RPMI-1640, DMEM, or DMEM/F12 media. However, when MCF10DCIS culture media is changed from RPMI-1640 to DMEM/F12, there is a noticeable reduction in cell proliferation rate. Additionally, care should be taken to maintain serum concentrations at a constant level because, without serum, colony formation will be inhibited[10]. Furthermore, after the agarose is microwaved, allow the bottle to equilibrate in a 45 °C water bath before mixing with media containing cells to prevent overheating the cells. Given that agarose solidifies rapidly at room temperature, we also recommend that all pipettes and 6-well culture plates are preheated in a 37 °C incubator before adding agarose solution to prevent premature solidification while handling.

While the soft agar colony formation technique is a valuable tool for measuring tumorigenicity in a range of cancer cell lines, some lines do not grow in soft agar. Another potential drawback of the method is that viable cells cannot be recovered once they are plated into the soft agar. Additionally, if a compound has a shorter bioavailability period, the feeding step will need to be performed more frequently (*i.e.* every other day) and the samples will need to be collected in less than seven days. The threshold for the colony size will also need to be reduced while still allowing for potential differences between the samples to be observed. In these instances, controls would need to be incorporated to optimize experimental parameters to minimize false-positive and false-negative results. Furthermore, this technique can be time consuming and difficult when testing a large number of samples. However, technological advances have helped to overcome some of these limitations. The conventional soft agar assay (as documented in this manuscript) typically uses 6-well culture plates or 6mm culture dishes. With automated plate

readers, however, multiple samples can be processed in 384-well plates[11]. For example, investigators pre-loaded tumor cells with dyes such as alamarBlue and tetrazolium dyes and colonies are quantified using plate reader, thus obviating the need to manually count colony number[11]. This high-throughput capability is, therefore, amenable for large scale cancer drug screens.

In sum, the soft agar assay is a valuable pre-clinical technique that can be used to assess the tumorigenicity of a wide-range of cancer cells (breast, prostate, ovarian, and others) with regards to their sensitivity to drugs, hormones, heat, hypoxia, and a multitude of other treatment conditions. The assay continues to provide a straightforward and informative tool for cancer researchers who wish to better understand the mechanisms of cancer progression and test the anti-tumor potential of new cancer therapies.

Disclosures

The authors have nothing to disclose.

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