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Isolation and characterization of methionine independent clones from methionine dependent cancer cells

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Abstract

Reversion to methionine independence is an extremely rare event in transformed and malignant cells. Unlike normal cells, transformed cells are unable to grow when methionine in the growth media is replaced with its metabolic precursor homocysteine. Methionine-independent revertants provide an excellent system to identify metabolic signatures and molecular characteristics associated with methionine dependency of transformed cells. Revertants maintain the genetic background and general growth behavior of the parental cell line, except they proliferate in methionine-free media supplemented with homocysteine. Here we describe a general approach to generate methionine-independent revertants using the example of the triple negative breast cancer cell line MDA-MB-468. To validate and characterize reversion we describe assays to evaluate cell proliferation and anchorage independent growth in soft agar.

Keywords

homocysteine; methionine stress resistance; methionine independence; anchorage independence

1. Introduction

Methionine metabolism is an essential metabolic pathway in mammals. Early studies of metabolism identified a strict metabolic requirement for the amino acid methionine in transformed, malignant cells known as the “methionine dependency of cancer”. When cultured in growth media where methionine has been replaced with its metabolic precursor homocysteine, malignant cells are unable to proliferate and undergo a distinct cell cycle arrest in G_1 or G2/M [1–7]. In contrast, proliferation of non-transformed, methionine independent cells is mostly unaffected by the metabolite replacement [8]. Reversion of transformed, methionine dependent cells to a non-transformed methionine independent state is a rare event, occurring approximately once in every 10,000 cells. Unlike their transformed counterparts, methionine-independent revertants can proliferate in homocysteine media indefinitely and exhibit normal-like cell properties including anchorage dependence [9].

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Derivation of methionine independent revertants from transformed cells was originally presented by Hoffman and colleagues in 1979 using SV40 transformed human cell lines and rat breast carcinoma fibroblast cells [9]. Here we describe a step by step procedure to isolate clonal methionine-independent cell lines derived from methionine-dependent cancer cells by prolonged culturing in homocysteine media (subheading 3.1). We have applied this strategy to the triple negative breast cancer cell line MDA-MB-468 but expect that this approach is applicable to other methionine-dependent cancer cell lines. The methionine independent revertants have similar proliferation rates in methionine supplemented growth media as the parental cell line, continue proliferating in homocysteine media, and have lost their tumorigenic hallmark for anchorage independent growth [5, 10].

To validate the spontaneous reversion of these clones, we describe methods for assessment of cell proliferation using the luminescent-based viability assay CellTiter-Glo® (Promega) (subheading 3.2), and anchorage independence using soft agar (subheading 3.3). The CellTiter-Glo® assay quantifies the amount of ATP from metabolically active cells, which is proportional to the number of cells in the culture. Since transformed, methionine dependent cells are unable to proliferate in homocysteine growth media, measuring proliferation rates in homocysteine media is the first step to evaluate revertant cell lines [5, 8, 10]. We and others have observed that reversion to methionine independence is typically accompanied by the loss of tumorigenic characteristics [10, 11]. While only xenograft assays can provide definitive information about the tumorigenic potential of revertant cell lines, a relatively simple evaluation of anchorage independent growth can be informative about tumorigenic properties without animal experiments. Anchorage independence is a well known signature of malignant cells and soft agar assays are a stringent method for transformation detection [12]. When suspended in soft agar, malignant cell growth is unaffected and colonies form in the agar layer, whereas non-transformed cells require attachment to a solid surface and do not proliferate. Anchorage independence assays are thus simple and effective methods to compare tumorigenic potential of methionine independent revertants from the transformed parental cell line.

Using a system of methionine dependent and independent cell pairs is ideal to identify unique metabolic signatures linking methionine dependence and tumorigenicity.

2. Materials

2.1 Deriving Methionine Independent Revertants from Methionine Dependent Transformed Cells

All procedures should be performed in a cell culture hood using sterile techniques and conditions. The method described uses the triple negative breast cancer cell line MDA-MB-468 and its specific growth conditions to generate methionine independent revertants.

2.1.1 Cell Culturing Materials

1. Methionine growth medium: Dulbecco’s Modified Eagle’s Medium with high glucose supplemented with 10% dialyzed fetal bovine serum (dFBS), 1.5 μM cyanocobalmin (vitamin B12), 4 mM L-glutamine, 100 μM L-cysteine, and 100

μM methionine (see Note 1). Filter with 0.22 μm membrane and store at 4 °C. Warm to 37 °C before use.

2. Homocysteine growth medium: Dulbecco's Modified Eagle's Medium with high glucose supplemented with 10% dialyzed FBS (dFBS), 1.5 μM cyanocobalmin (vitamin B12), 4 mM L-glutamine, 100 μM L-cysteine, and 370 μM DL-homocysteine (see Note 2). Filter with 0.22 μm membrane and store at 4 °C. Warm to 37 °C before use.

3. PBS (phosphate buffered saline): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4. In 1 L graduated cylinder or glass beaker with a stir bar, add 800 mL distilled water and stir at medium speed. Weigh 8 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, and 0.24 g KH_2PO_4 and add to stirring water. After all salts have dissolved adjust the pH to 7.4 with HCl. Add distilled water to a total volume of 1 L. Autoclave and store at room temperature.

4. Cell culture grade trypsin.

5. 150 mm, 24-well, 6-well, and 100 mm cell culture plates

6. 200 μL micropipettor with sterile tips

2.2 Luminescent Measurement of Cell Viability

The majority of transformed, malignant cell lines are methionine dependent and cannot proliferate when cultured in homocysteine media. In comparison, non-transformed and primary cells can utilize homocysteine and continue to proliferate when methionine is not available in the growth media. To determine if a cell line is methionine independent, we routinely use the luminescent cell viability assay CellTiter-Glo® (Promega), which provides a quick read-out for cell proliferation (see Note 3).

2.2.1 Materials

1. Methionine growth media (described in 2.1.1)

2. Homocysteine growth media (described in 2.1.1)

3. PBS (described in 2.1.1)

4. Cell culture grade trypsin

5. 96-well white plates with clear plastic bottom

6. CellTiter-Glo® luminescent viability assay (Promega): Reconstitute reagent per manufacture's instructions. Aliquot 10 mL in 15 mL conical tubes. Store at −20 °C.

7. Luminometer platereader

2.3 Assessment of Anchorage Independence with Soft Agar

All procedures should be prepared in sterile conditions as much as possible using sterile reagents, media, and glassware. The following protocol provides instructions for preparing ten 0.35% soft agar assays in 35 mm Petri dishes.

2.3.1 Base Agar

1. 1% w/v low gelling temperature agar: prepare a minimum of 10 mL 1% low gelling temperature agar in sterile water. In a sterilized glass bottle with a stir bar, warm 10 mL sterile water to 37 °C. With the stir bar on medium speed, add 100 mg low gelling temperature agar to warm water and stir until completely dissolved. Keep agar in 40 °C water bath for immediate use (see Note 4).
2. 2x Methionine growth media: 1x methionine growth media is described in 2.1.1. Prepare 10 mL growth media with twice the amount of additives including FBS and antibiotics (see Note 5). Warm to 40 °C in water bath.
3. 35 mm sterile Petri dishes (see Note 6).

2.3.2 Top Agar Layer with Cell Suspension

1. 0.7% w/v low gelling temperature agar: prepare a minimum of 10 mL 0.7% low gelling temperature agar in sterile water. In a sterilized glass bottle with a stir bar, warm 10 mL sterile water to 40 °C. With the stir bar on medium speed, add 70 mg low gelling temperature agar to warm water and stir until completely dissolved. Keep agar in 40 °C water bath for immediate use (see Note 7).
2. 1x Methionine growth media for preparation of cell suspension (see Note 5).
3. 2x Methionine growth media: 1x methionine growth media is described in 2.1.1. Prepare 10 mL growth media with twice the amount of additives including FBS and antibiotics (see Note 5). Warm to 40 °C in water bath.
4. PBS (described in 2.1.1)
5. Hemocytometer to prepare cell suspension.
6. Cell culture grade trypsin for adherent cell suspensions.
7. Serum containing growth media to collect cells after trypsin induced cell dissociation.
8. Cell suspension of 6,667 cells per 1 mL in 2x growth media (see Notes 8, 9, and 10).

2.3.3 Assay Maintenance

1. 1x Methionine growth media warmed to 37 °C (described in 2.1.1, see Note 5).

2.3.4 Staining Colonies

1. 0.005 % crystal violet solution prepared in 50% ethanol.
2. PBS

3. Methods

3.1 Deriving Methionine Independent Revertants from Methionine Dependent Transformed Cells

1. Culture cells in a 150 mm cell culture plate in methionine growth media until they reach 80% confluency.
2. Remove and discard methionine growth media and add 10 mL pre-warmed (37 °C) PBS to the plate to wash the cell monolayer. Swirl plate to remove all traces of methionine and aspirate PBS wash.
3. Perform a second 10 mL PBS wash and aspirate to discard.
4. Add 20 mL pre-warmed homocysteine growth media to the cell culture plate and return cells to humidified incubator at 37 °C.
5. In the first week, replace the media every day to remove detached cells. Aspirate the current growth media and wash once with 10 mL pre-warmed PBS. Replenish the cells with 20 mL pre-warmed homocysteine media.
6. In the second week, replace the media every 2 days to remove detached cells and replenish media components.
7. Use a microscope to identify areas of growing cells.
8. Continue to culture cell colonies until they are large enough to be visualized without a microscope.
9. Once the colonies can be visualized without a microscope cells can be isolated and transferred to a 24-well plate.
10. Prepare a 24-well plate for cell transfer by filling wells with 500 μL pre-warmed homocysteine growth media.
11. Remove and discard growth media of cell culture plate containing cell colonies.
12. Wash once with 10 mL PBS and aspirate to discard.
13. Add 10 mL pre-warmed homocysteine growth media to cell culture plate to prevent cell dehydration during colony isolation.
14. To collect a single colony, hold the plate at a 45 ° angle to clear the media from the region of interest.
15. Using a micropipettor with a sterile tip gently touch the tip to the cell colony and lift. (see Note 11)
16. Transfer colony to 24-well plate and gently resuspend to mix (see Note 12).
17. Place cells in humidified incubator at 37 °C and allow selected clones to grow.
18. When cells have grown to about 80% confluency, use standard subculturing methods to dissociate cells from 24-well plate and replate in 6-well plates (see Note 9).

19. At this point, cells can be switched back to methionine growth media or maintained in homocysteine media for further selection (see Note 5).

20. Continue to culture cells and expand clonal cell lines as necessary.

3.3 Luminescent Measurement of Cell Viability

This assay is performed over the course of several days. Each 96-well plate is collected for each time point. We typically perform this assay over six days, collecting time points at 0, 2, 4, and 6 days and thus we require four identically plated 96-well plates. To ensure data quality, we plate at least 4 wells per treatment for each time point.

1. From cultured cells, prepare a cell suspension to be plated into 96-well white plates with a clear plastic bottom (see Note 9).

2. Plate 2,000–10,000 cells per well in each 96-well plate (see Note 13). Allow the cells to attach to the plate for at least 16 hours before proceeding.

3. Wash the cells twice with 200 μL PBS per well and aspirate all residual liquid from each well (see Note 14).

4. Add 100 μL of either methionine or homocysteine growth media and return all plates to the cell culture incubator at 37 °C except for your day 0 plate—see below (see Note 15).

5. To collect each time point, remove the plate from the cell culture incubator and place directly in a −80 °C freezer (see Note 16).

6. Thaw an adequate amount (100 μL/well) of CellTiter-Glo® reagent for 1 hour at room temperature.

7. Remove samples from −80 °C and immediately add 100 μL/well of CellTiter-Glo® (see Note 17 and 18).

8. Allow the plate to reach room temperature before proceeding to luminometer platereader. The reaction is stable for 1 hour (see Note 19).

3.3 Assessment of Anchorage Independence with Soft Agar

3.3.1 Preparing the Base Agar Layer

1. 0.5% agar base: in 50 mL conical tube, gently combine 10 mL of 1% agar and 10 mL 2x complete growth media until fully mixed while preventing bubbles in the solution. This will prepare 20 mL of 0.5% agar.

2. Add 1.5 mL of 0.5% agar base to each 35 mm Petri dish. Gently swirl to ensure the bottom of each dish is completely covered. The remaining 0.5% agar can be discarded.

3. Set dishes aside for a minimum of 30 minutes at room temperature to allow agar to solidify (see Note 20).

3.3.2 Preparing the Top Agar Layer

1. If the dishes with the base layer were prepared ahead of time, remove from 4 °C and allow to equilibrate to room temperature for at least 30 minutes.
2. Label dishes with base agar appropriately for samples and positive and negative controls (see Note 21).
3. For blank assays in triplicate, mix 2.25 mL 0.7% agar with 2.25 mL 2x growth media in 15 mL conical tube. Gently mix to avoid the introduction of bubbles. Pipette 1.5 mL onto the top of three dishes containing a base layer.
4. For cell suspension assays in triplicate, add 2.25 mL 0.7% agar to 15 mL conical containing 2.25 mL cell suspension in 2x growth media. Gently mix to avoid the introduction of bubbles and damage to cells. Pipette 1.5 mL into three dishes with base layer.
5. Place dishes at 4 °C for 30 minutes to solidify (see Note 22).
6. Incubate assays at 37 °C in humidified incubator for 10 to 30 days.

3.3.3 Assay Maintenance

1. Add 0.75 mL of 1x growth media to plates 1 or 2 times per week to prevent the agar from dehydrating.
2. Ensure the incubator’s humidity by maintaining water levels if necessary. A lack of humidity will cause the assays to dehydrate resulting in cell death.

3.3.4 Staining Colonies

1. Aspirate any remaining media from the dish without disturbing the agar by holding the dish at a 45 degree angle allowing the media to collect on the side of the dish.
2. Add 0.5 mL of 0.005% crystal violet for a minimum of 1 hour. Gently agitate dishes with a rocker on a low setting at room temperature. (see Note 23)
3. Aspirate the crystal violet solution from the dishes without disturbing the agar.
4. Quickly add 0.5 mL 1x PBS to dish, swirl, and aspirate. Do not leave the dish in PBS, the agar will soften and become to difficult to handle (see Note 25).
5. Count colonies using a dissecting microscope or scan.

4. Notes

1. The media described is the recommended culturing condition for MDA-MB-468 cells, this should be modified for the cell line of interest. Dialyzed fetal bovine serum (dFBS) must be used to eliminate FBS as a source for methionine and other amino acids. Our DMEM of choice is high glucose without glutamine, cysteine, methionine, or homocysteine and thus these components must be supplemented.

2. Homocysteine can be readily oxidized and must be stored at −20 °C. We prepare homocysteine media in small portions of 100 mL and discard any excess media after 1 week. For our experiments we use DL-homocysteine, however, L-homocysteine is commercially available. If L-homocysteine is used the final concentration should be 185μM.

3. A number of alternative assays such as MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) or sulforhodamine B can also be used [13, 14].

4. It is best to use the prepared soft agar immediately but it can be stored at room temperature and melted again for future use. Properly seal glass bottle to prevent water evaporation, which increases agar density. When agar is reheated, maintain in water bath for 30 minutes to lower the temperature before use.

5. For standard culturing and anchorage independence assays it is unnecessary to use dFBS in the growth medium because methionine dependence is not being assessed. Generally dFBS is more expensive and we only use it prior to and during experiments in which cells cultured in methionine and homocysteine growth media are being compared.

6. Assays can be prepared in 6-well plates instead of 35 mm Petri dishes. However, we have found that assays in 6-well plates evaporate at different rates depending on the location of the well in the plate and thus we recommend using individual dishes for each assay.

7. Certain cell types are sensitive to the percentage of top agar; determine if 0.3% or 0.4% agar is best for the cell type of interest. In our assays for MDA-MB-468 cells we choose to use a final concentration of 0.35% agar.

8. The number of cells used per assay needs to be optimized. We found that 5,000 cells per assay is optimal for MDA-MB-468 cells.

9. We use trypsin to release the adherent cells from the cell culturing surface. First, aspirate current media and wash cells with pre-warmed PBS to remove all traces of serum. Aspirate PBS and add pre-warmed trypsin to cover cell monolayer, swirl plate to ensure trypsin is evenly distributed. We use 0.5% trypsin and generally need to incubate our cells for 10 min at 37 °C; however, trypsin percentage and incubation times will vary depending on trypsin potency, cell type, and cell density and therefore incubation time should be monitored and kept to a minimum. Collect cells by adding serum containing growth medium and gently pipette up and down to break up any clumps. Transfer cell suspension to a 15 mL conical tube and centrifuge (300 x g, 5 minutes, room temperature). Aspirate the supernatant and resuspend pellet thoroughly in 1x methionine growth media.

10. Cell suspensions should be prepared as normally done during subculturing or as described in Note 9. After centrifugation, aspirate the supernatant and resuspend pellet thoroughly in 1x methionine growth media to prepare for cell counting. Count the resuspended cells using a hemocytometer. For 3 assays using 5,000 cells per assay we need 2.25 mL of 15,000 cells (5,000 cells/0.75 mL or 6,667

cells/mL). Transfer the equivalent of 15,000 cells to a new 15 mL conical tube. Centrifuge, aspirate supernatant, and resuspend cell pellet in 2.25 mL 2x methionine growth media. Adjust accordingly for quantity of assays and cell number.

11. If the cells do not lift off the plate easily you can gently slide the pipette tip from one side of the colony to the other. This may cause cell damage and should not be used as a first option. Alternatively, cloning cylinders can be used to harvest cells using trypsin-induced dissociation.

12. It is fine if the colony does not completely break up into a single cell suspension, the cells will still adhere and grow in the 24-well plate. Trypsin will be used in a later steps to disrupt the colony and produce a single cell suspension during replating.

13. Plating number depends on cell size. For example, MDA-MB-468 cells are smaller than MDA-MB-231 and thus we use 8,000 cells/well of MDA-MB-468 and 3,000 cells/well MDA-MB-231.

14. It is crucial to remove all of the residual methionine from the growth media and PBS washes before treating with homocysteine media.

15. To compare proliferation rates of each cell line throughout the time course, each plate should have the same cell line treated in both methionine and homocysteine growth media.

16. These samples can be stored in −80 °C up to one month.

17. When using an electric multichannel pipette, always use 5 mL more CellTiter-Glo® then calculated to ensure the bottom of the reagent reservoir is covered. Unused reagent can be recycled and stored in −20 °C.

18. A significant reduction in luciferase signal occurs when the reagent is added to cells kept at room temperature for an extended period before adding the CellTiter-Glo® reagent.

19. The luciferase in the CellTiter-Glo® reagent mixture utilizes ATP from sample cells to produce luminescent signals as cell viability per cell number readout. If the experimental procedure severely affects ATP production it will be inadequate to use CellTiter-Glo® to measure cell viability.

20. These plates can be stored up to 1 week at 4 °C. Stack plates, wrap in foil, and store upside down for storage. For best results, prepare the base layer 1 to 2 days in advance before adding top layer.

21. We recommend plating each cell line in replicates of no less than three. Including a positive control and an assay without cells is recommended. Positive controls should be an anchorage independent tumorigenic cell line, we have used the triple negative breast cancer cell line MDA-MB-468. An assay with top and base layers without cells is helpful for a background control and also monitors any contamination.

22. Placing the assays at 4 °C will ensure the agar is fully solidified before incubating at 37 °C and will not harm the cells. In our experience, if the assays do not completely solidify at room temperature before placing in the incubator, the agar will remain in a semi-solid state and the assay will be difficult to work with for quantification.

23. An alternative for colony staining option is nitro-blue tetrazolium chloride.

24. When staining the assay it is important to not disturb the agar. Having been at room temperature, treated with crystal violet, and washed with PBS makes the agar soft and easily damaged or aspirated into a vacuum if touched.

5. References

1. Chello PL, Bertino JR (1973) Dependence of 5 methyltetrahydrofolate utilization by L5178Y murine leukemia cells in vitro on the presence of hydroxycobalamin and transcobalamin II. Cancer Res 33:1898–1904. [PubMed: 4737200]
2. Halpern BC, Clark BR, Hardy DN, et al. (1974) The effect of replacement of methionine by homocystine on survival of malignant and normal adult mammalian cells in culture. Proc Natl Acad Sci U S A 71:1133–1136. doi: 10.1073/pnas.71.4.1133 [PubMed: 4524624]
3. Hoffman RM, Erbe RW (1976) High in vivo rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine. Proc Natl Acad Sci U S A 73:1523–1527. doi: 10.1073/pnas.73.5.1523 [PubMed: 179090]
4. Stern PH, Wallace CD, Hoffman RM (1984) Altered methionine metabolism occurs in all members of a set of diverse human tumor cell lines. J Cell Physiol 119:29–34. [PubMed: 6707100]
5. Booher K, Lin DW, Borrego SL, Kaiser P (2012) Downregulation of Cdc6 and pre-replication complexes in response to methionine stress in breast cancer cells. Cell Cycle 11:4414–4423. [PubMed: 23159852]
6. Lin DW, Chung BP, Kaiser P (2013) S-adenosylmethionine limitation induces p38 mitogen-activated protein kinase and triggers cell cycle arrest in G1. J Cell Sci 127:50–59. [PubMed: 24155332]
7. Hoffman RM, Jacobsen SJ (1980) Reversible growth arrest in simian virus 40-transformed human fibroblasts. ProcNatlAcadSciUSA 77:7306–7310.
8. Mecham JO, Rowitch D, Wallace CD, et al. (1983) The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. Biochem Biophys Res Commun 117:429–434. doi: 10.1016/0006-291X(83)91218-4 [PubMed: 6661235]
9. Hoffman RM, Jacobsen SJ, Erbe RW (1979) Reversion to methionine independence in simian virus 40-transformed human and malignant rat fibroblasts is associated with altered ploidy and altered properties of transformation. Proc Natl Acad Sci U S A 76:1313–1317. [PubMed: 220612]
10. Borrego SL, Fahrmann J, Datta R, et al. (2016) Metabolic changes associated with methionine stress sensitivity in MDA-MB-468 breast cancer cells. Cancer Metab 4:9. doi: 10.1186/s40170-016-0148-6 [PubMed: 27141305]
11. Hoffman RM, Jacobsen SJ, Erbe RW (1978) Reversion to methionine independence by malignant rat and SV40-transformed human fibroblasts. Biochem Biophys Res Commun 82:228–234. doi: 10.1016/0006-291X(78)90600-9 [PubMed: 208554]
12. Mori S, Chang JT, Andrechek ER, et al. (2010) An anchorage-independent cell growth signature identifies tumors with metastic potential. 28:2796–2805. doi: 10.1038/onc.2009.139.An
13. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63. doi: 10.1016/0022-1759(83)90303-4 [PubMed: 6606682]
14. Skehan P, Storeng R, Scudiero D, et al. (1990) New Colorimetric Cytotoxicity Assay for. 1107–1112.

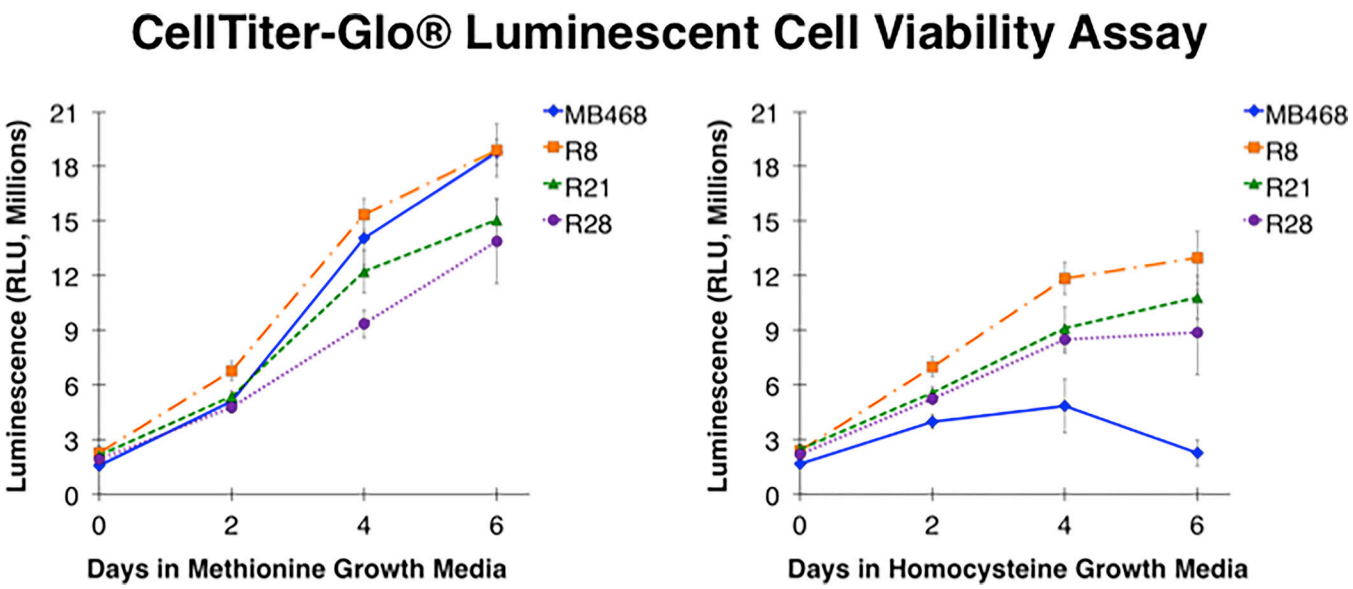

Figure 1.

MDA-MB-468 cancer cells and the methionine independent revertant clones MDA-MB-468res-R8, -21, and -R28 were cultured in either methionine or homocysteine growth media. Samples were collected at 0, 2, 4, and 6 days and treated with CellTiter-Glo®. Unlike the methionine independent revertants, the cancer cell line MDA-MB-468 cannot proliferate in homocysteine growth media.

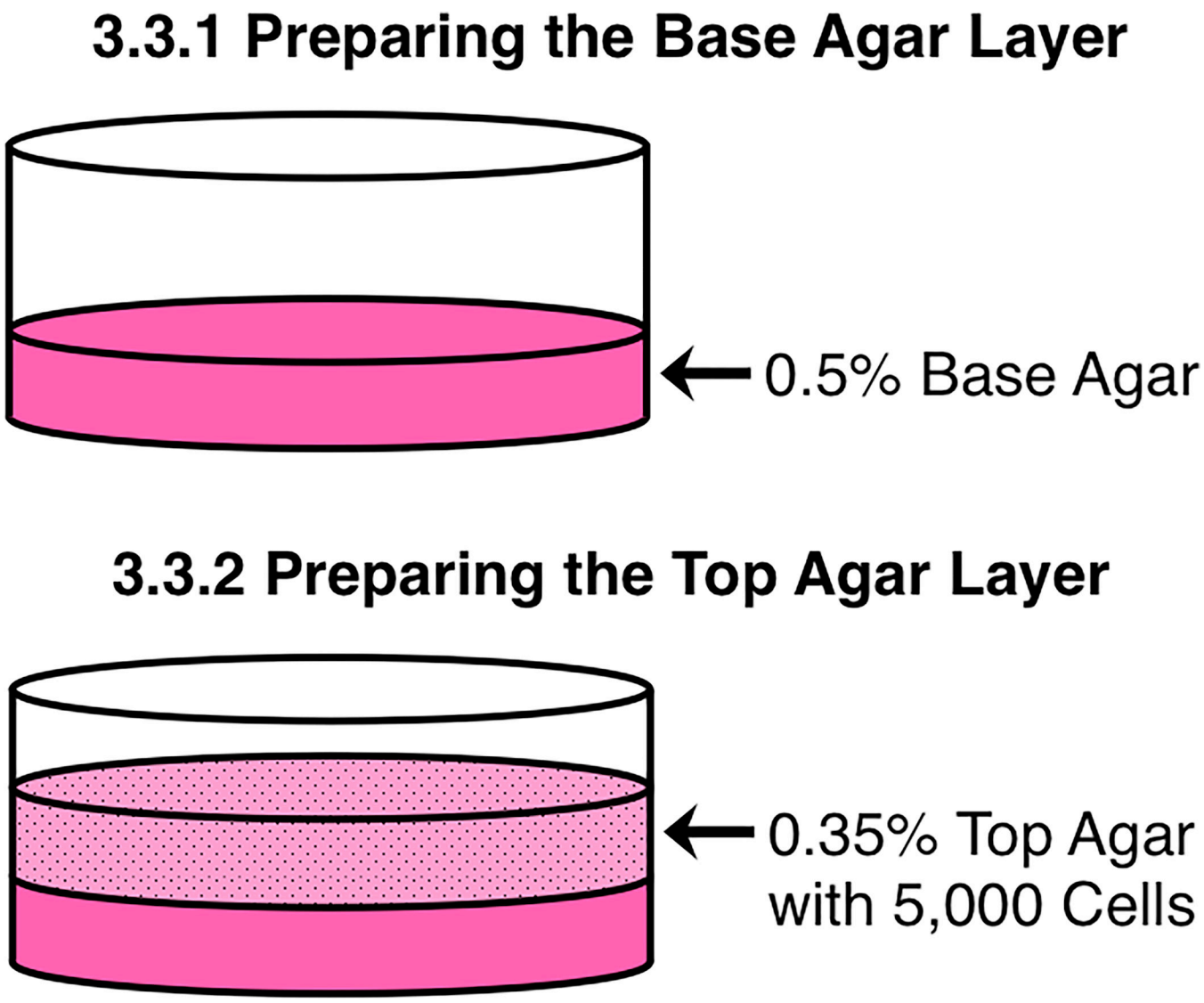

Figure 2.
Soft agar assays are a stringent method for cell transformation detection. Each assay is composed of two layers, a lower 0.5% agar base layer (described in 3.3.1) and an upper 0.35% agar top layer containing the cells of interest (described in 3.3.2).

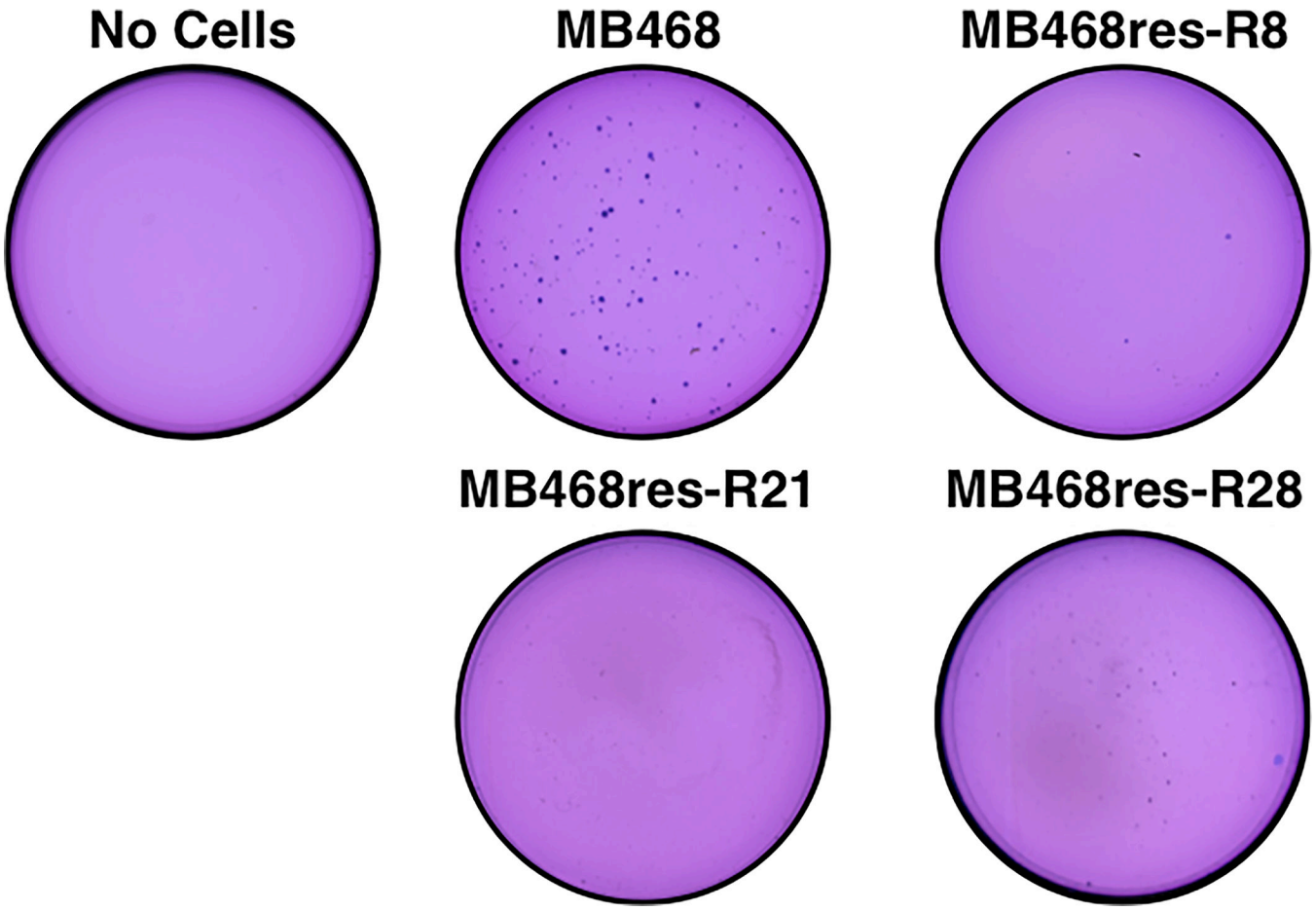

Figure 3.

Methionine independent revertant clones MDA-MB-468res-R8, -R21, and -28 form fewer and smaller clones in soft agar as compared to the parental MDA-MB-468 cell line. Control plate does not contain cells.