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Title: Mammosphere Assay Reveals Api5-Induced Stemness in Non-Tumorigenic Breast Epithelial Cell Lines

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

Authors: 2.9 and 3.9 do not have to be filmed under a microscope. These are just normal scenes where the talent is working with a microscope

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations? **No**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 22

Number of Shots: 45

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

REQUIRED: ~~Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Snehal Bhatia:** We try to understand how overexpression of a protein in a non-tumorigenic cell line leads to the acquisition of cancer stem cell-like properties.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Snehal Bhatia:** This protocol uses a minimal medium supplemented with B27 and EGF, making it more cost-effective.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.3. **Snehal Bhatia:** A challenge in the protocol is standardizing the cell count to ensure sufficient mammosphere formation without cell aggregation.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Snehal Bhatia:** Mammospheres generated by this protocol can also be used to visualise protein localisation in a three-dimensional context using immunofluorescence.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Seeding Primary Generation Mammospheres

Demonstrator: Snehal Bhatia

- 2.1. To begin, coat each well of a standard 6-well tissue culture plate with 1 milliliter of 1.2 percent Poly-2-hydroxyethyl methacrylate solution [1] and place the plate in an oven set at 40 degrees Celsius to dry under sterile conditions [2].
 - 2.1.1. Talent dispensing 1 milliliter of pHEMA into each well of a 6-well plate using a pipette.
 - 2.1.2. Talent placing the pHEMA-coated plate inside an oven labeled “40 °C” in a sterile environment.
- 2.2. Expose the dried plate to ultraviolet light for 15 minutes [1]. Then, wash each well three times with Dulbecco’s PBS before using the plate to set up the mammosphere formation assay [2].
 - 2.2.1. Talent placing the plate under an ultraviolet light source for timed exposure.
 - 2.2.2. Talent washing each well of the plate three times using Dulbecco’s PBS.
- 2.3. Before cell seeding, remove the spent medium from the culture dish [1]. Wash the cells with 1 milliliter of Dulbecco’s PBS [2]. Then, add 500 microliters of 0.05 percent Trypsin-EDTA [3] and incubate at 37 degrees Celsius in a humidified 5 percent carbon dioxide incubator for 12 to 15 minutes until the cells are trypsinized [4].
 - 2.3.1. Talent aspirating spent medium from the culture dish.
 - 2.3.2. Talent pipetting 1 milliliter of Dulbecco’s PBS into the culture dish to wash cells.
 - 2.3.3. Talent adding 500 microliters of 0.05 percent Trypsin-EDTA to the dish.
 - 2.3.4. Talent placing the dish inside a labeled carbon dioxide incubator set to 37 degrees Celsius.
- 2.4. Once the cells are dislodged, add 2 milliliters of resuspension medium to neutralize the trypsin activity [1]. Spin the cell suspension at 112 g for 10 minutes at room temperature [2].
 - 2.4.1. Talent pipetting 2 milliliters of resuspension medium into the dish containing

dislodged cells.

- 2.4.2. Talent placing the tube with cell suspension into a centrifuge and setting it to 112 g for 10 minutes.
- 2.5. Aspirate the supernatant [1] and resuspend the resulting cell pellet in 1 milliliter of mammosphere medium [2]. Pass the cell suspension through a 25-gauge needle to create a single-cell suspension [3].
 - 2.5.1. Talent aspirating the supernatant from the centrifuge tube.
 - 2.5.2. Talent pipetting 1 milliliter of mammosphere medium to resuspend the cell pellet. **AUTHOR'S Note: Divided into 2 parts**
 - 2.5.3. Talent drawing the suspension into a syringe fitted with a 25 gauge needle and passing it through to ensure single-cell dispersion.
- 2.6. Next, using a hemocytometer, count the number of cells [1]. Calculate the required volume of the cell suspension to seed 2,000 cells per well [2].
 - 2.6.1. Talent loading the cell suspension into a hemocytometer
 - 2.6.2. Talent viewing through a microscope.
- 2.7. Dilute the calculated volume of cell suspension in 2 milliliters of mammosphere medium per well [1] and seed the diluted suspension into each well of a 6-well plate, preparing three replicate wells for each condition [2].
 - 2.7.1. Talent adding the calculated volume of cell suspension into a tube with 6 milliliters of mammosphere medium.
 - 2.7.2. Talent pipetting 2 milliliters of the prepared suspension into triplicate wells of a 6-well plate.
- 2.8. Incubate the plate at 37 degrees Celsius in a humidified 5 percent carbon dioxide incubator for 7 days [1].
 - 2.8.1. Talent placing the seeded 6-well plate into a labeled carbon dioxide incubator set to 37 degrees Celsius.
- 2.9. After 7 days, manually count the number of mammospheres larger than 50 micrometers in each well [1]. Image the mammospheres using a digital camera attached to a light microscope at 20x magnification [2-TXT].

2.9.1. Talent observing wells under a microscope and counting mammospheres.

AUTHOR'S NOTE: 2.9.2 is clubbed with this take.

2.9.2. Talent operating the system to obtain images. **TXT: Calculate the mammosphere formation efficiency**

3. Seeding Secondary Generation Mammospheres

3.1. Collect the first-generation mammospheres in a 15-milliliter tube [1] and centrifuge at 300 g for 10 minutes at room temperature [2].

3.1.1. Talent transferring mammospheres from the wells into a 15 milliliter centrifuge tube.

3.1.2. Talent placing the tube into a centrifuge and setting it to 300 g for 10 minutes.

3.2. Aspirate the supernatant carefully, leaving the mammosphere pellet intact at the bottom of the tube [1]. Wash the pellet with Dulbecco's PBS [2]. Centrifuge the cell suspension to obtain a mammosphere pellet [3] then pipette out the Dulbecco's PBS without disturbing the pellet [4].

NOTE: The VO has been edited to accommodate the added shots

3.2.1. Talent aspirating the supernatant without disturbing the mammosphere pellet.

3.2.2. Talent pipetting Dulbecco's PBS into the centrifuge tube containing the pellet and gently mixing.

Added shot: Centrifuge the cell suspension to obtain a mammosphere pellet.

Added shot: Aspirate Dulbecco's PBS from the tube without disturbing the

3.3. Now, add 200 microliters of 0.05 percent Trypsin-EDTA to the washed pellet [1] and incubate at 37 degrees Celsius in a humidified 5 percent carbon dioxide incubator for 2 to 3 minutes [2]. Then, pass the mammospheres in trypsin solution through a 25-gauge needle three times to obtain a single-cell suspension [2].

3.3.1. Talent pipetting 200 microliters of 0.05 percent Trypsin-EDTA into the tube.

3.3.2. Talent placing the tube in a carbon dioxide incubator set to 37 degrees Celsius.

3.3.3. Talent drawing the trypsinized mammosphere solution into a syringe with a 25 gauge needle and passing it through.

- 3.4. Then, add 0.5 milliliters of resuspension medium to neutralize the trypsin activity [1]. Centrifuge the cell suspension at 112 g for 10 minutes at room temperature [2].
 - 3.4.1. Talent pipetting 0.5 milliliters of resuspension medium into the cell suspension tube.
 - 3.4.2. Talent placing the tube in a centrifuge and setting it to 112 g for 10 minutes.
- 3.5. Aspirate the supernatant [1] and resuspend the cell pellet in 0.5 milliliters of mammosphere medium [2].
 - 3.5.1. Talent aspirating the supernatant from the centrifuge tube.
 - 3.5.2. Talent pipetting 0.5 milliliters of mammosphere medium into the tube to resuspend the pellet.
- 3.6. Using a hemocytometer, count the number of cells [1] and calculate the required volume of cell suspension to seed 2,000 cells per well [2].
 - 3.6.1. Talent loading the cell suspension into a hemocytometer.
 - 3.6.2. Talent observing through a microscope.
- 3.7. Dilute the required cell suspension volume in 2 milliliters of mammosphere medium per well [1] and seed into each well of a 6-well plate, preparing three replicate wells for each condition [2].
 - 3.7.1. Talent adding the calculated cell suspension volume to 6 milliliters of mammosphere medium in a tube.
 - 3.7.2. Talent pipetting the suspension into each well of a 6-well plate in triplicate.
- 3.8. Incubate the plate at 37 degrees Celsius in a humidified 5 percent carbon dioxide incubator for 7 days [1].
 - 3.8.1. Talent placing the 6-well plate into a labeled carbon dioxide incubator set at 37 degrees Celsius.
- 3.9. After 7 days, count the number of mammospheres with a diameter greater than 50 micrometers in each well [1-TXT].
 - 3.9.1. Talent examining wells under a microscope and counting mammospheres.
TXT: Image the mammospheres; Calculate the self-renewal capacity

4. Sample Preparation for Western Blotting to Detect the Changes in Pluripotency Markers

- 4.1. Collect the first-generation mammospheres into a 15-milliliter tube [1] and centrifuge at 300 *g* for 10 minutes at room temperature [2].
 - 4.1.1. Talent transferring mammospheres into a 15 milliliter centrifuge tube using a pipette.
 - 4.1.2. Talent placing the tube in a centrifuge and setting it to 300 *g* for 10 minutes.
- 4.2. Aspirate the supernatant carefully, ensuring the mammosphere pellet remains undisturbed at the bottom of the tube [1]. Wash the pellet gently with Dulbecco's PBS [2].
 - 4.2.1. Talent aspirating the supernatant without disturbing the pellet.
 - 4.2.2. Talent adding Dulbecco's PBS to the tube and gently mixing to wash the pellet.
- 4.3. Collect the washed pellet in ice-cold RIPA buffer to obtain the protein lysate [1] and perform immunoblotting using the standard protocol [2].
 - 4.3.1. Talent transferring the pellet into a tube containing ice-cold RIPA buffer.
 - 4.3.2. Talent preparing and running the immunoblotting setup with labeled protein samples.
- 4.4. Finally, quantify the protein levels using densitometric analysis in ImageJ [1]. Select the region of interest on the Western blot image and generate a density profile for each lane and the pluripotency marker [2-TXT].
 - 4.4.1. Show the ImageJ interface with the Western blot image loaded and the region of interest tool being used to outline specific bands.
 - 4.4.2. Display the **Plot Profile** window showing generated density profiles for each selected lane. **TXT: Normalize the relative protein densities to GAPDH levels; Calculate the fold change for each protein**

Results

5. Results

- 5.1. Api5-overexpressing MCF10A cells showed a 1.58-fold increase in mean mammosphere formation efficiency [1] compared to control cells under non-adherent, serum-deprived conditions [2].
 - 5.1.1. LAB MEDIA: Figure 1BC. *Video editor: Highlight the image and violin plot for "Api5 OE" group.*
 - 5.1.2. LAB MEDIA: Figure 1BC. *Video editor: Highlight the image and violin plot for "Control"*
- 5.2. These cells formed second-generation mammospheres with a self-renewal capacity [1] nearly 2.5 times higher than control cells [2].
 - 5.2.1. LAB MEDIA: Figure 1DE. *Video editor: Highlight the image and violin plot for "Api5 OE" group.*
 - 5.2.2. LAB MEDIA: Figure 1DE. *Video editor: Highlight the image and violin plot for "control" group.*
- 5.3. Immunoblotting showed increased expression of Oct3/4 (oct-3-4), Nanog, Sox2, and Bmi1 (B-M-I-1) in Api5-overexpressing primary mammospheres [1] compared to controls [2].
 - 5.3.1. LAB MEDIA: Figure 2AB. *Video editor: Highlight the four bands labeled "Oct 3/4," "Nanog," "Sox2," and "Bmi1" under "Api5 OE" lanes.*
 - 5.3.2. LAB MEDIA: Figure 2AB. *Video editor: Highlight the four bands labeled "Oct 3/4," "Nanog," "Sox2," and "Bmi1" under "Control".*
- 5.4. Densitometric analysis showed a 1.83-fold increase in Oct3/4 [1], a 1.65-fold increase in Nanog [2], a 1.68-fold increase in Sox2 [3], and a 1.62-fold increase in Bmi1 levels in Api5-overexpressing mammospheres [4] compared to controls [5].
 - 5.4.1. LAB MEDIA: Figure 2E. *Video editor: Highlight the bar labeled "Api5 OE" showing a higher fold change in "Oct 3/4 levels"*
 - 5.4.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the bar labeled "Api5 OE" showing a higher fold change in "Nanog levels"*
 - 5.4.3. LAB MEDIA: Figure 2D. *Video editor: Highlight the bar labeled "Api5 OE"*

showing a higher fold change in “Sox2 levels”

- 5.4.4. LAB MEDIA: Figure 2F. *Video editor: Highlight the bar labeled “Api5 OE” showing a higher fold change in “Bmi1 levels”*
- 5.4.5. LAB MEDIA: Figure 2CDEF *Video editor: Highlight the four bars labeled “Control”*

Pronunciation Guide:

1. Mammosphere
Pronunciation link: <https://www.merriam-webster.com/dictionary/mammosphere>
IPA: /'mæməˌsfɪr/
Phonetic Spelling: mam·uh·sfeer
2. Tumorigenic
Pronunciation link: <https://www.merriam-webster.com/dictionary/tumorigenic>
IPA: /ˌtuːməˈrɪdʒənɪk/, /ˌtjuːməˈrɪdʒənɪk/
Phonetic Spelling: too·muh·rij·uh·nik, tyoo·muh·rij·uh·nik
3. Epithelial
Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>
IPA: /ˌepəˈθiːliəl/
Phonetic Spelling: ep·uh·thee·lee·uhl
4. Overexpression
Pronunciation link: <https://www.merriam-webster.com/dictionary/overexpression>
IPA: /ˌoʊvərɪkˈspresjən/
Phonetic Spelling: oh·ver·ik·spresh·uhn
5. Stemness
Pronunciation link: No confirmed link found
IPA: /'stɛm.nəs/
Phonetic Spelling: stem·nuhs
6. Immunofluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/immunofluorescence>
IPA: /ɪˌmjuːnoʊflʊˈresəns/
Phonetic Spelling: ih·myoo·noh·flor·es·uhns
7. Methacrylate
Pronunciation link: <https://www.merriam-webster.com/dictionary/methacrylate>
IPA: /məˈθækrəˌleɪt/
Phonetic Spelling: muh·thak·ruh·layt
8. Dulbecco
Pronunciation link: <https://www.howtopronounce.com/dulbecco>
IPA: /dʊlˈbɛkoʊ/
Phonetic Spelling: dul·bek·oh
9. Trypsin
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /ˈtrɪpsɪn/
Phonetic Spelling: trip·sin

10. Centrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /ˈsentrɪˌfjuːdʒ/

Phonetic Spelling: sen-truh-fyooj

11. Hemocytometer

Pronunciation link: <https://www.merriam-webster.com/dictionary/hemocytometer>

IPA: /ˌhiːməsaɪˈtɑːmətər/

Phonetic Spelling: hee-moh-sy-tom-uh-ter

12. Resuspension

Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspension>

IPA: /ˌriːsəˈspɛnʃən/

Phonetic Spelling: ree-suh-spen-shuhn

13. Pluripotency

Pronunciation link: <https://www.merriam-webster.com/dictionary/pluripotent>

IPA: /ˌplʊrɪˈpoutəntsi/

Phonetic Spelling: ploor-ih-poh-tuhn-see

14. Immunoblotting

Pronunciation link: <https://www.merriam-webster.com/dictionary/immunoblot>

IPA: /ɪˌmjuːnoʊˈblɑːtɪŋ/

Phonetic Spelling: ih-myoo-noh-blot-ing

15. Densitometric

Pronunciation link: <https://www.merriam-webster.com/dictionary/densitometry>

IPA: /ˌdensɪˈtɑːmɛtrɪk/

Phonetic Spelling: den-sih-tah-ma-trik

16. Densitometric

Pronunciation link: <https://www.merriam-webster.com/dictionary/densitometry>

IPA: /ˌdensɪˈtɑːmɛtrɪk/

Phonetic Spelling: den-sih-tah-ma-trik

17. Densitometry

Pronunciation link: <https://www.merriam-webster.com/dictionary/densitometry>

IPA: /ˌdensɪˈtɑːmɛtri/

Phonetic Spelling: den-sih-tom-uh-tree

18. Api5

Pronunciation link: No confirmed link found

IPA: /ˌɛpiːˈaɪ faɪv/

Phonetic Spelling: ay-pee-eye five

19. Nanog

Pronunciation link: <https://www.howtopronounce.com/nanog>

IPA: /ˈnænoʊ/

Phonetic Spelling: nan·og

20. Sox2

Pronunciation link: <https://www.howtopronounce.com/sox2>

IPA: /sɒks tuː/

Phonetic Spelling: soks two

21. Bmi1

Pronunciation link: <https://www.howtopronounce.com/bmi1>

IPA: /ˌbiːɛmˈaɪ wʌn/

Phonetic Spelling: bee·em·eye one

22. Densitometric

Pronunciation link: <https://www.merriam-webster.com/dictionary/densitometry>

IPA: /ˌdɛnsɪˈtɑːmɛtrɪk/

Phonetic Spelling: den·sih·tah·ma·trik