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Title: In Vitro Evaluation of Oncogenic Transformation in Human Mammary Epithelial Cells

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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 similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: all screen captures provided; do not film

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **54**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Anna Genescà**: This methodology uses the integration of different indicators to evaluate the degree of transformation of any cell model **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Anna Genescà**: This technique provides researchers with a set of time-consuming but simple-to-use tools for evaluating cell transformation in vitro. This allows the method to be performed in most laboratories **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator on Camera

1.3. **Anna Genescà**: Demonstrating the procedure will be Teresa Anglada, a Postdoctoral researcher from my laboratory, and Joan Repullés, a Microscopy Specialist **[1][2]**.

1.3.1. INTERVIEW: Author saying the above

1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera NOTE: 2 shots for this: 1.3.2 Teresa and 1.3.2 Joan

Protocol

2. Breast Primary Epithelial Cell Passaging and Population Doubling Calculation

2.1. When the breast primary epithelial cell culture reaches 90% confluency **[1]**, transfer the supernatant from the culture flask to a 15-milliliter tube containing 2 milliliters of fetal bovine serum **[2]** and wash the cells with PBS **[3]**.

2.1.1. LAB MEDIA: 2.1.1. BPECs 90 confluency

2.1.2. WIDE: Talent transferring supernatant from flask to tube, with supernatant container visible in frame

2.1.3. Talent washing cells, with PBS container visible in frame

2.2. Treat the cells with 1 milliliter of 3x trypsin for 5 minutes at 37 degrees Celsius **[1]**. When the cells have detached **[2]**, inactivate the trypsin with the reserved medium **[3]** and collect the cells by centrifugation **[4-TXT]**.

2.2.1. Talent adding trypsin to flask, with trypsin container visible in frame, then talent carrying the flask towards the incubator NOTE: should be an additional shot for this

2.2.2. LAB MEDIA: 2.2.2. BPEC suspension trypsinization

2.2.3. Reserved medium being added to flask

2.2.4. Talent placing tube into centrifuge **TEXT: 5 minutes, 500 g, RT**

2.3. Flick the tube to resuspend the pellet **[1]** and add 1-2 milliliters of fresh medium for counting **[2]**.

2.3.1. Shot of pellet if visible, then supernatant removal and tube being flicked

2.3.2. Talent adding medium to tube, with medium container visible in frame

2.4. After determining the cell concentration, add 3 x 10^5 cells to a new tube **[1]** and bring the final volume to 5 milliliters with additional fresh culture medium **[2]**.

2.4.1. Talent adding cells to tube

2.4.2. Talent adding medium to cells, with medium container visible in frame

2.5. Seed cells into a new T-25 flask **[1]** and return the cells to the cell culture incubator **[2]**.

2.5.1. Talent adding the mixture to flask
2.5.2. Talent placing flask into incubator

2.6. Then use the formula to calculate the number of accumulated population doublings **[1-TXT]**.

2.6.1. BLACK TEXT ON WHITE BACKGROUND: $PD = PD_i + \log (N_h/N_s) / \log_2$

2.7. To assess the proliferation rate of the cells, plot the accumulated number of population doublings for a specific time interval **[1]**. An increased slope indicates an increased cell proliferation rate **[2]**.

2.7.1. LAB MEDIA: Figure 4
2.7.2. LAB MEDIA: Figure 4 *Video Editor: please sequentially emphasize Partially and Fully transformed data lines*

3. Three-Dimensional Cell Culture in Basement Membrane Matrix and Immunofluorescent Protein Detection

3.1. To set up a 3D cell culture, rinse wells with a 1.9-square centimeter surface area with cold sterile PBS **[1-TXT]** and coat the bottom of each well with 100 microliters of basement membrane matrix **[2]**.

3.1.1. WIDE: Talent rinsing wells with PBS, with PBS container visible in frame *Videographer: Important step* **TEXT: Materials and reagents must be cold**
3.1.2. Talent adding basement membrane matrix to well(s), with basement membrane matrix container visible in frame *Videographer: Important step*

3.2. Then place the plate in the cell culture incubator for about 20 minutes to allow the matrix to solidify **[1]**.

3.2.1. Talent placing plate into incubator Author NOTE: We made a mistake when numbering this shot on the clapper-board. We wrote 3.1.3, but the correct number is 3.2.1.

3.3. In the meantime, harvest mammary cells as just demonstrated **[1]** and dilute the cells to a 4×10^5 cells/milliliter concentration in medium **[2]**.

3.3.1. Talent adding cells to eppendorf, with empty flask visible in frame
3.3.2. Talent adding medium to eppendorf, with medium container visible in frame

3.4. Mix the cells with fresh medium supplemented with 8% basement membrane matrix at a 1:1 ratio **[1]** and add 500 microliters of the cells to each well of solidified matrix **[2]**.

3.4.1. Talent mixing medium with 8% basement membrane matrix and cells in an eppendorf, with cell and matrix containers visible in frame *Videographer: Important step*
3.4.2. Talent adding cells to well(s) *Videographer: Important step*

3.5. Incubate the cells at 37 degrees Celsius for a few minutes **[1]** before adding 500 microliters of medium supplemented with 4% basement membrane matrix to each well **[2]**.

3.5.1. Talent placing plate into incubator
3.5.2. Talent adding medium with basement membrane matrix to well(s), with medium + matrix container visible in frame

3.6. Place the cells in the cell culture incubator for 14 days **[1-TXT]**. The seeded cells will group and proliferate, forming acini-like structures **[2]**.

3.6.1. Talent placing plate into incubator **TEXT: Refresh medium 2-3x/wk**
3.6.2. LAB MEDIA: 3.5.2. Timelapse

3.7. For the immunofluorescent protein detection in the 3D-cultured mammary cells, use a cutoff P200 pipette tip to remove the basement membrane matrix from each well **[1]** and smear approximately 50 microliters of disaggregated matrix from each well onto individual glass slides **[2]**.

3.7.1. Talent removing matrix *Videographer: Important step*
3.7.2. Matrix being smeared onto slide *Videographer: Important step*

3.8. When the samples have completely dried, fix with the cells with a 1:1 methanol-acetone solution at minus 20 degrees Celsius for 30 minutes **[1]**. After fixing, block samples with blocking solution for 2 hours at room temperature **[2-TXT]**.

3.8.1. Talent adding slide(s) to methanol:acetone container
3.8.2. Talent adding slide(s) to blocking solution container **TEXT: Blocking solution: 5% normal goat serum + 0.1% triton-X-100 in PBS**

3.9. At the end of the incubation, add 30 microliters of the primary antibody working solution of interest **[1]** and cover the slides with a strip of laboratory wrapping film for an overnight incubation at 4 degrees Celsius in a humid chamber **[2]**.

3.9.1. Talent adding antibody to slide(s), with antibody container(s) visible in frame
3.9.2. Talent covering slide with wrapping film and placing slide in a humid chamber

3.10. The next morning, wash the slides three times with PBS for 1 hour per wash **[1]** and label the samples with the appropriate secondary antibody solution as just demonstrated **[2]**.

3.10.1. Talent adding slides to PBS container
3.10.2. Talent adding antibody to slide without PBS, with antibody container(s) visible in frame

3.11. At the end of the incubation, wash the samples two times in PBS for 2 hours per wash **[1]** before counterstaining with DAPI (DAP-ee) diluted in antifade mounting medium **[2]** and covering each slide with a coverslip **[3]**.

3.11.1. Talent adding slides to PBS container
3.11.2. Talent adding mounting medium to slide(s), with mounting medium container visible in frame
3.11.3. Coverslip being placed

3.12. Then analyze the fluorescent signal distribution for each *acinus* on a confocal microscope **[1-TXT]**.

3.12.1. Talent at microscope, looking at slide **TEXT: See text for suggested microscope imaging parameter details**

4. Anchorage-Independent Assay and Colony Quantification

4.1. To perform an anchorage-independent assay, first cover the bottom of the appropriate number of wells of a 6-well plate with 1.5 milliliters of 0.6% agar in medium solution **[1]** and let the agar solidify at room temperature **[2]**.

4.1.1. WIDE: Talent adding medium to well(s), with medium container visible in frame
4.1.2. Shot of solidified agar

4.2. Next, prepare a 5 x 10^4 cells/milliliter solution as demonstrated **[1]** and filter the cells through a 40-micron strainer into a sterile 50-milliliter tube **[2]**.

4.2.1. Talent adding cells to tube, with empty flask visible in frame *Videographer: Important step*
4.2.2. Talent adding cells to filter *Videographer: Important step*

4.3. Holding the tube at a 45-degree angle, add an equal volume of 0.6% agar in medium solution down the wall of the tube **[1]** and, after thorough mixing, layer 1 milliliter of the cells in agar suspension to each well of the previously prepared 6-well plate **[2]**.

4.3.1. Shot of tilted tube, then agar solution being added to cells *Videographer: Important/difficult step*
4.3.2. Talent adding cells to plate *Videographer: Important step*

4.4. Use an inverted microscope to confirm that the cells have been homogenously dispersed throughout the agar **[1-TXT]**.

4.4.1. LAB MEDIA: 5.4.1. Cells agar **TEXT: Discard and repeat seeding if cell clumping**

4.5. When the agar has completely solidified, carefully add 1 milliliter of fresh medium to each well without disturbing the agar **[1]** and incubate the cells for 3 weeks changing the medium twice per week **[2-TXT]**.

4.5.1. Talent adding medium to plate
4.5.2. Talent placing plate into incubator **TEXT: Refresh medium 2x/wk**

4.6. At the end of the colony formation period, replace the medium with 1 milliliter of 1 milligram/milliliter MTT (M-T-T) per well for a 24-hour incubation in the cell culture incubator **[1]**.

4.6.1. Talent adding MTT to well(s), with MTT container visible in frame

4.7. The next day, remove the MTT from each well **[1]** and acquire images of each well on the inverted microscope **[2]**.

4.7.1. MTT being removed
4.7.2. Talent at microscope, imaging cells

4.8. When all of the images have been acquired, open the images in ImageJ **[1]** and click **Image**, **Adjust**, **Threshold,** and **Apply** to obtain a binary mask through thresholding of the original image **[2-TXT]**.

4.8.1. Talent at computer, opening image(s), with monitor visible in frame
4.8.2. SCREEN: Screenshot_1: 00:08-00:19 **TEXT: Automatic processing and analysis macro provided**

4.9. When well-delimited colonies have been obtained, select **Plugins** and **BioVoxxel** to launch the **Extended Particle Analyzer** to identify the MTT positive colonies **[1-TXT]**.

4.9.1. SCREEN: Screenshot_2: 00:02-00:14 **TEXT: Suggested initial colony parameters: Size (micron^2) = 250-Infinity; Solidity = 0.75-1**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
3.1., 3.4., 3.7., 4.2., 4.3.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.3. To ensure success tilt the cell containing tube about 45 degrees and drop the agar through the internal wall of the tube. This will allow the agar solution to cool down just enough to not damage the cells but preventing its premature solidification.

Results

5. Results: Representative BPEC Transformation Evaluation

5.1. Over a specific interval of time, partially **[1]** and fully transformed cells achieve a higher number of population doublings compared to non-transformed cells **[2]**, indicating that the cell division rate is increased with the transformation process **[3]**.

5.1.1. LAB MEDIA: Figure 4 *Video Editor: please add/emphasize bracket between Partially and Non-transformed data lines*
5.1.2. LAB MEDIA: Figure 4 *Video Editor: please add/emphasize bracket between Fully and Non-transformed data lines*
5.1.3. LAB MEDIA: Figure 4

5.2. Over the 2-week 3D cell culture period, the cells aggregate and proliferate, increasing the *acini* size **[1]**.

5.2.1. LAB MEDIA: Figure 5B

5.3. The proper polarization of each *acinus* can be accurately assessed using protein immunofluorescent detection in conjunction with three-dimensional signal location by confocal microscopy **[1]**.

5.3.1. LAB MEDIA: 7.3.1. Polarized acinus

5.4. While all of the *acini* formed by non-transformed breast cells are properly organized **[1]**, a loss of polarization is observed in *acini* formed by partially and fully transformed cells **[2]**.

5.4.1. LAB MEDIA: Figure 6 *Video Editor: please emphasize Figure 6Ai and N data bar*
5.4.2. LAB MEDIA: Figure 6 *Video Editor: please emphasize Figure 6Aii and D and T data bars*

5.5. After 3 weeks of embedded agar culture **[1]**, cells with an anchorage-independent growth capacity give rise to colonies composed of multiple cells **[2]**.

5.5.1. LAB MEDIA: Figure 8
5.5.2. LAB MEDIA: Figure 8 *Video Editor: please 3 week image row*

5.6. Only metabolically active cells are capable of cleaving the tetrazolium MTT ring, resulting in purple MTT formazan crystals after 24 hours **[1]**.

5.6.1. LAB MEDIA: Figure 7B *Video Editor: please emphasize ring*

5.7. Measurement of the colony diameter by automatic image analysis **[1]** allows the filtering of low proliferative colonies or individual cells **[2]**.

5.7.1. LAB MEDIA: Figure 7C *Video Editor: please emphasize steps i, ii, iii and iv sequentially*

5.7.2. LAB MEDIA: Figure 9 *Video Editor: please emphasize comparison of size between first row and Single cell MTT +*

5.8. The number of colonies formed by non-transformed cells is negligible compared to those from partially and fully transformed cells **[1]**. Colony size can also be used to discriminate between cells with different degrees of transformation **[2]**.

5.8.1. LAB MEDIA: Figure 10 *Video Editor: please emphasize white dots, then dark gray and black dots. Please highlight "Number" row in Figure 10A*

5.8.2. LAB MEDIA: Figure 10 *Video Editor: please highlight "Diameter" row in 10A*

Conclusion

6. Conclusion Interview Statements

6.1. **Anna Genescà**: These assays can be used to screen cell lines prior to animal inoculation, as only those cell lines that have tested positive in vitro should be inoculated into mice **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera