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TITLE:

In Vitro Evaluation of Oncogenic Transformation in Human Mammary Epithelial Cells

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KEYWORDS:

cellular transformation, growth curve, anchorage assay, 3D cell culture, human mammary epithelial cells, tumorigenesis

SUMMARY:

This protocol provides experimental in vitro tools to evaluate the transformation of human mammary cells. Detailed steps to follow-up cell proliferation rate, anchorage-independent growth capacity, and distribution of cell lineages in 3D cultures with basement membrane matrix are described.

ABSTRACT:

Tumorigenesis is a multi-step process in which cells acquire capabilities or hallmarks that allow their growth, survival, and dissemination under hostile conditions. Different tests seek to identify and quantify these hallmarks of cancerous cells; however, they often focus on a single aspect of cellular transformation and, in fact, multiple tests are required for their proper characterization. The purpose of this work is to provide researchers with a set of tools to assess cellular

transformation in vitro from a broad perspective, thereby making it possible to draw sound conclusions.

A sustained proliferative signaling activation is the major feature of tumoral tissues and can be easily monitored under in vitro conditions by calculating the number of population doublings achieved over time. Besides, the growth of cells in 3D cultures allows their interaction with surrounding cells, resembling what occurs in vivo. This enables the evaluation of cellular aggregation and, together with immunofluorescent labeling of distinctive cellular markers, to obtain information on another relevant feature of tumoral transformation: the loss of proper organization. Another remarkable characteristic of transformed cells is their capacity to grow without attachment to other cells and to the extracellular matrix, which can be evaluated with the anchorage assay.

Detailed experimental procedures to evaluate cell growth rate, to perform immunofluorescent labeling of cell lineage markers in 3D cultures, and to test anchorage-independent cell growth in soft agar are provided. These methodologies are optimized for Breast Primary Epithelial Cells (BPEC) due to its relevance in breast cancer; however, procedures can be applied to other cell types after some adjustments.

INTRODUCTION:

Multiple successive events are required for neoplasm development. In 2011, Hanahan and Weinberg described 10 capabilities that enable transformed cells' growth, survival, and dissemination: the so-called "Hallmarks of Cancer"[1]. The methodology described here compiles three different tools to evaluate in vitro cellular transformation by focusing on some of the tumoral cells' distinctive features. These techniques assess the cell proliferation rate, the behavior of cells when cultured in 3D and their capacity to form colonies with anchorage independence.

Cell models are crucial to test hypothesis in vitro. Different approaches have been developed to generate experimental models of cellular transformation for the study of cancer[2–4]. Since breast cancer is the most common cancer among women worldwide and is responsible for approximately 15% of cancer deaths[5] among women, providing suitable cellular models of mammary epithelial cells is of utmost importance for further investigation. In this article, we have illustrated the potential of three techniques to evaluate cellular transformation using an experimental model of Breast Primary Epithelial Cells (BPECs) transformation initially described by Ince and colleagues in 2007[6] and later implemented in our laboratory[7]. This experimental model is based on the sequential alteration of three targeted genes (SV40 Large T and small t antigens herein referred to as *Ttag*, *hTERT*, and *HRAS*) to the genome of non-transformed BPECs. Moreover, the method used for BPECs derivation favors the maintenance of mammary epithelial cells with luminal or myoepithelial markers, resulting in a heterogeneous cell culture that retains some of the mammary gland physiological traits.

In the mammary gland, luminal mammary epithelial cells, which are responsible for milk production, are located near the lumen, whereas myoepithelial cells are disposed around luminal

cells and take care of contraction movements leading the milk to the nipple. The loss of proper organization between these cell lineages is a feature of tumoral transformation[8] that can be assessed in vitro after immunofluorescent detection of distinctive lineage markers in 3D cell cultures. Another major characteristic of tumoral cells is their capacity to grow without attachment to other cells and to the extracellular matrix[1]. When healthy cells are forced to grow in suspension, mechanisms such as *anoikis* – a type of cell death induced in response to detachment from the extracellular matrix – are activated[9]. The evasion of cell death is one of the distinctive hallmarks of cancer and thus, transformed cells are capable to inactivate *anoikis* and survive in an anchor-independent manner. This capacity can be evaluated in vitro with the anchorage-independent assay using soft agar. Furthermore, an inherent feature of tumoral tissues is their sustained proliferative signaling capacity, which can be easily monitored under in vitro conditions by measuring the increase of cell number along time, not only in suspension assays but also by monitoring the growth rate of monolayer adherent cultures.

Despite the best model to test tumorigenic potential is the inoculation of tumoral cells in murine models and evaluation of tumor development in situ, it is important to minimize the number of animals employed in experimental procedures as much as possible. Therefore, having suitable tests to assess transformation in vitro is a top priority. Here, we provide a set of tools to evaluate the tumorigenic potential of partially and fully transformed breast epithelial cells that can be easily implemented in most of the laboratories that work with cellular transformation models.

PROTOCOL:

Human samples used in the following experiments were obtained from reduction mammoplasties carried out at *Clínica Pilar Sant Jordi* (Barcelona) under standard procedure consent. All procedures are performed in a Class II Biological Safety Cabinet unless otherwise stated.

1. In vitro culture of human mammary epithelial cells and growth curve plot build-up

1.1. In vitro culture of breast primary epithelial cells (BPECs): cell passaging

NOTE: For BPEC derivation and cell culture follow instructions described by Ince et al.[6].

1.1.1. Medium preparation.

1.1.1.1. Supplement WIT basal defined medium with P or T supplements, provided by the manufacturer, depending on whether primary or transformed BPECs are cultured.

1.1.1.2. Add cholera toxin to the supplemented WIT medium to a final concentration of 100 ng/mL for primary or 25 ng/mL for transformed BPECs.

CAUTION: Cholera toxin is fatal if swallowed. Use personal protective equipment. Avoid its release to the environment.

1.1.2. Cell culture maintenance and passaging.

NOTE: For the following steps keep in mind that cells are growing in a T25 flask. Nonetheless, volumes can be adapted to other cell culture formats maintaining the proportionality in terms of surface area.

1.1.2.1. Check on the cell confluency every day. When the culture is 90% confluent, perform cell passaging.

1.1.2.2. Acquire 1x PBS, 3x trypsin, medium and a 15 mL conical tube containing 2 mL of Fetal Bovine Serum (FBS) for each flask.

1.1.2.3. Remove the medium from the flask and keep it in the 15 mL conical tube containing FBS.

1.1.2.4. Rinse cells with 1x PBS.

1.1.2.5. Detach cells from the surface by adding 1 mL of 3x trypsin. Incubate for 5 min at 37 ˚C.

1.1.2.6. Check if cells have been detached. Apply vigorous shaking if cells are not completely detached.

1.1.2.7. Inactivate trypsin by adding the reserved medium supplemented with FBS.

1.1.2.8. Harvest the cellular suspension and place it in the 15 mL conical tube.

1.1.2.9. Centrifuge at 500 *x g*, eliminate the supernatant, and resuspend the pelleted cells by flicking the bottom of the tube with a finger.

1.1.2.10. Add 1–2 mL of fresh media to the pellet and measure the cell concentration using an automatic cell counter or a hemocytometer. This data will later be used to calculate the population doublings and draw the growth curve. Seed 12,000 cells/cm^2 (e.g., 300,000 cells for a T25 flask) in modified cell culture surface flasks (see **Table of Materials**).

NOTE: Dilute the cell suspension solution if the concentration is too high to ensure proper quantification.

1.1.2.11. Add medium to a final volume of 5 mL and incubate the cells at 37 ˚C and 5% CO_2 atmosphere.

1.1.2.12. Replace the cell culture medium every 48 h.

1.2. Population doubling time calculation and data visualization

1.2.1. Using data of the cell count obtained in the step 1.1.2.10, apply the following formula to obtain the accumulated population doubling (PD) values:

$$PD = PD_i + log\ (N_h / N_s) / log2 \quad (1)$$

Where, PD_i denotes the number of population doublings achieved by the cells until the previous subculture (it refers to the PD accumulated on the previous subculture), N_h is the number of harvested cells, and N_s is the number of seeded cells.

1.2.2. Represent data for a specific interval of time using an XY graph where the number of days in culture (*x*-axis) and the accumulated PD (*y*-axis) are represented.

1.2.3. Get the best-fit line and the fitting equation:

$$y = bx + a \quad (2)$$

NOTE: An increased slope (b) means an increased proliferation rate.

2. Three-dimensional (3D) culture in basement membrane matrix and immunofluorescent protein detection

2.1. 3D culture in basement membrane matrix

NOTE: This protocol has been adapted from Debnath et al., 2003[10] and is optimized for 24 well plates (see **Table of Materials**).

2.1.1. Prepare the material a day before the experiment: pre-chill basement membrane matrix overnight at 4 ˚C and let pipette tips, microcentrifuge tubes, and well plates cool in the freezer.

NOTE: The matrix must be kept at -20 ˚C for long-term storage. Make aliquots to avoid multiple freeze-thaw cycles.

2.1.2. On the day of the experiment, place pre-cooled material on ice.

2.1.3. Rinse wells with cold sterile 1x PBS in order to reduce surface tension.

2.1.4. Cover the bottom of each well with 100 µL of basement membrane matrix.

NOTE: Dispense the matrix slowly and spread it throughout the well; it is crucial to avoid bubble formation in the bottom layer to avert monolayer cell culture growth.

2.1.5. Place the plate in the incubator, at 37 ˚C, to let the matrix layer solidify.

NOTE: It usually takes about 20 min to solidify.

2.1.6. Meanwhile, trypsinize cells as explained previously in step 1.1. Centrifuge the cells at 500 x *g* for 5 min and resuspend in medium. Prepare a 400,000 cells/mL suspension and gently disaggregate any cell clump by pipetting.

2.1.7. Prepare the medium with 8% basement membrane matrix and mix 1:1 (v/v) with cellular suspension to obtain a 200,000 cells/mL solution in 4% matrix.

NOTE: Calculate the amount of medium needed to avoid matrix unnecessary waste.

2.1.8. Place 500 µL of cell suspension in matrix solution on top of the already solidified matrix layer to seed a total amount of 100,000 cells in medium with 4% basement membrane matrix.

2.1.9. Incubate cells at 37 ˚C for a few minutes and then, add 500 µL of the medium with 4% basement membrane matrix. Incubate the cells at 37 ˚C in an incubator with 5% CO_2 for 14 days. Seeded cells will group and proliferate to originate the *acini*-like structures.

NOTE: Cell motility and aggregation can be monitored by time-lapse during the 3D formation process. Use image analysis software (e.g., Fiji/ImageJ or Imaris) to evaluate these events. The number and size of the *acini* depend on the aggregation process and the proliferation rate and might vary between cell types. Adjust basement membrane matrix concentration and seeded cells to obtain desired 3D structures.

2.1.10. Add 500 µL of the medium with 4% basement membrane matrix 2–3 times per week.

NOTE: Avoid disturbance of the layers carrying the plate gently during manipulation.

2.1.11. If desired, the number and size of *acini* can be measured during the culture period. To do so, take random pictures at different times after seeding using a phase contrast or DIC inverted microscope. Use image analysis software to measure the diameter of 100–200 3D structures.

2.2. Immunostaining

NOTE: Sterile conditions are not required during this part of the protocol.

2.2.1. Remove the culture medium.

2.2.2. Tear the basement membrane matrix using a p200 pipette tip with the end cut off. Place ~50 µL of disaggregated matrix on top of a glass slide and smear it in an area of 1–2 cm^2.

2.2.3. Let the sample dry completely at room temperature or use a heating plate at 37 ˚C to accelerate the process. Fix samples with methanol:acetone (1:1, v/v) at -20 ˚C for 30 min.

NOTE: Fluorescent signal of previous markers, such as that of fluorescent proteins expressed by

the cells, will be erased.

CAUTION: Methanol is flammable, toxic if inhaled, swallowed or in case it comes in contact with skin. Wear personal protective equipment and work inside a fume hood.

2.2.4. Discard the fixation solution and remove the excess, if any, by reclining the slide on filter paper.

NOTE: The protocol can be paused here. Once dry, slides can be stored at -20 ˚C for several months.

2.2.5. Block samples epitopes with 5% normal goat serum and 0.1% triton-X-100 in 1x PBS (blocking solution) for 2 h at room temperature.

2.2.6. Meanwhile, prepare antibodies working solutions by diluting primary or secondary antibodies at the desired concentration in blocking solution.

NOTE: Antibody concentration must be accurately adjusted depending on the cell type and the antibody reference. As a guide, to identify cells from luminal and myoepithelial lineages in BPEC, primary anti-Cytokeratin 14 and anti-Claudin-IV antibodies (see **Table of Materials**) can be used. The recommended working solution concentration is 1:100 for these primary antibodies and 1:500 for anti-Mouse and anti-Rabbit secondary antibodies (see **Table of Materials**).

2.2.7. Add 30 µL of primary antibodies working solution and cover it with a strip of laboratory wrapping film to avoid evaporation. Incubate overnight at 4 ˚C in a humid chamber. Wash three times with 1x PBS for 1 h each.

2.2.8. Repeat step 2.2.7 for secondary antibodies. Incubation should be performed in darkness.

2.2.9. Wash with 1x PBS for 2 h.

NOTE: Adjust antibodies concentration, incubation time, and washing hardness to improve the signal/noise ratio for specific samples.

2.2.10. Remove the remaining PBS and, once dry, counterstain with DAPI at 0.25 µg/mL diluted in antifade mounting medium. Cover slides with a coverslip by letting it settle without applying pressure. Seal with nail polish.

NOTE: Samples can be stored at 4 ˚C for several weeks. For long-term storage, keep them at -20 ˚C.

2.2.11. Analyze fluorescent signal distribution for each *acinus* using a confocal microscope.

NOTE: Confocal microscope configuration must be accurately determined depending on the equipment used and the antibodies applied to the sample. As a guide, equipment and reagents are detailed in the **Table of Materials**.

2.2.12. Use a 40x objective and the following laser and detector settings: for DAPI use excitation with a 405 laser (3%–5%), detection with a PMT detector (800V, Offset: -9) and a spectral band from 410 nm to 500 nm; for A488 (Claudin-IV) use excitation with a 488 laser (7%–10%), detection with a PMT detector (800V, Offset: -20) and a spectral band from 490 nm to 550 nm; and for Cy3 (Cytokeratin 14) use excitation with a 555 laser (2%–10%), detection with a PMT detector (800V, Offset: -35) and a spectral band from 560 nm to 600 nm.

3. Anchorage-independent assay, MTT staining and automatic colony quantification

3.1. Anchorage-independent assay: agar and cellular suspension plating

NOTE: Protocol has been adapted from Borowicz et al., 2014[11] to perform experiments in BPECs.

3.1.1. Prepare a 1.2% agar solution diluted in ultrapure water in a sterile bottle. Autoclave the solution and maintain it at 42 ˚C during the experiment. The agar solution can be stored at 4 ˚C; when required, heat the agar solution until it is liquid again.

CAUTION: Use heat-resistant gloves to avoid burn after autoclave.

NOTE: From now on, sterile conditions must be maintained.

3.1.2. Prepare a 0.6% agar solution by mixing 1:1 (v/v) complete pre-warmed medium with 1.2% agar solution. Maintain at 42 ˚C to avoid premature solidification.

NOTE: Medium can be previously double supplemented to obtain a fully supplemented 0.6% agar + medium solution once mixed.

3.1.3. Cover the bottom of a 35 mm well with 1.5 mL of 0.6% agar in medium solution and let it solidify at room temperature. Make sure that the bottom of the plate is completely covered before agar solidification, otherwise, cells may adhere to the plate and grow in monolayer.

NOTE: Adherent and non-adherent surface plates can be used.

3.1.4. Meanwhile, trypsinize cells and, once centrifuged and resuspended in medium, prepare a 50,000 cells/ml solution and gently disaggregate any cell clump by pipetting repeatedly.

3.1.5. Prepare a 0.3% agar + cell suspension in the medium at a final concentration of 25,000 cells/mL.

NOTE: Optimal cell concentration may differ among cell types. Try different concentrations until

individualized colonies are formed.

3.1.5.1. Place a 40 µm strainer filter on top of a 50 mL sterile tube and filter the 50,000 cells/mL solution letting it drop into the bottom of the tube.

3.1.5.2. Remove the filter from the 50 mL sterile tube, tilt the cell-containing tube about 45 ˚C and drop the same volume of 0.6% agar + medium solution pouring it through the internal wall of the tube. This will allow the agar solution to cool down just enough to not damage the cells and avoid its premature solidification.

3.1.6. Homogenize the mixture and deposit 1 mL of 0.3% agar + cell suspension in the medium (containing 25,000 cells) on top of the previously solidified bottom agar layer.

3.1.7. Visualize seeded cells using an inverted microscope to make sure that the cells are individualized. Otherwise, the experiment should be repeated.

3.1.8. Wait until the agar layer is completely solidified, then carefully add 1 mL of the fresh medium on top without disturbing the delicate agar layers beneath.

3.1.9. Incubate the cells at 37 ˚C and 5% CO_2 in an incubator for 3 weeks.

NOTE: The time required for colony formation can vary among different cell types, but usually 3 weeks are sufficient.

3.1.10. Change medium twice per week. To do so, gently tilt the plate toward you, aspirate medium in the lower corner, and add 1 mL of fresh medium.

NOTE: Avoid touching the agar layers as they easily detach from the plate.

3.2. MTT staining

3.2.1. Prepare Thiazolyl Blue Tetrazolium Bromide (MTT) stock solution at 6 mg/mL in ultrapure water in a sterile bottle and filter solution using 0.2 µm filters. This MTT solution can be stored for up to 6 months at -20 ˚C.

CAUTION: MTT may cause irritation and is suspected of causing genetic defects. Use safety glasses, gloves, and a respiratory filter.

NOTE: Avoid repeated freeze-thaw cycles.

3.2.2. Prepare a working solution of MTT at 1 mg/mL by diluting the stock solution with sterile ultrapure water.

3.2.3. Once the colony formation period has concluded, remove the medium from the plate and add 1 mL of 1 mg/mL MTT to each well.

3.2.4. Incubate for 24 h in the incubator. Remove the MTT solution by aspirating it gently. Plates can be stored at 4 ˚C for several weeks.

NOTE: Avoid light exposure to prevent non-specific crystal formation.

3.3. Colony quantification

3.3.1. Obtain images of each plate using an inverted microscope. Adjust the magnification in order to acquire the maximum field of view with fewer images and still being able to detect small colonies (usually 4x or 10x objectives).

NOTE: Make sure that images present a homogeneous background. Neither phase-contrast nor differential interference contrast is required since non-stained colonies will not be quantified.

3.3.2. Upload images to ImageJ/Fiji software[12] to count the number of colonies and the area of each MTT-positive colony.

NOTE: The script for automatic quantification is provided as a **Supplementary File**. To execute the code, paste it to the macro editor (**Plugins | New | Macro**) and follow the instructions.

3.3.2.1. Obtain a binary mask through thresholding the original image (**Image | Adjust | Threshold**) to obtain well-delimited colonies.

NOTE: 8- or 16-bit images are usually required to perform this step. The "Minimum threshold" method is recommended.

[Place Figure 1 here]

3.3.2.2. Run the Extended Particle Analyzer from Biovoxxel plugin[13] (**Plugins | BioVoxxel | Extended Particle Analyzer**) to identify MTT positive colonies. Initial guiding conditions: Size (μm^2) = 250–Infinity; Solidity = 0.75–1.00.

[Place Figure 2 here]

3.3.3. Estimate the average diameter (D) from each colony area value (A) according to the formula:

$$D = 2\,(A/\pi)^{½} \qquad (3)$$

3.3.4. Filter results by excluding low proliferative colonies (e.g., low diameter).

3.3.4.1. Choose a minimum number of divisions per week (*m*) to be considered (e.g., 1).

3.3.4.2. Estimate the radius of a colony (*R*) with *n* cells according to the following formula:

$$R = r\,(n/\rho)^{1/3} \qquad (4)$$

Where, *r* is the average radius of individual cells in suspension, *n* is the number of cells forming a colony that suffered *m* divisions each week during *w* weeks in culture. In exponential growth: $n = 2^{(m*w)}$. ρ is the Packaging efficiency. Note that, in random movement, packaging efficiency is ~0.64 and the densest possible packing fraction for identical spheres is 0.74[14].

3.3.4.3. Discard all colonies that present a diameter lower than 2R as their cells have not achieved the minimum number of divisions considered in step 3.3.4.1.

REPRESENTATIVE RESULTS:

An experimental model of cellular transformation with the introduction of three genetic elements in BPECs was chosen to generate representative results of oncogenic transformation[6,7] (**Figure 3**). Non-transformed BPECs (***N***) were derived from disease-free breast tissue as described by Ince and colleagues[6] and cultured following the protocol indicated here. After overcoming STASIS (stress or aberrant signaling induced-senescence, a phenomenon typically observed in mammary epithelial cells in vitro that is overcome around 4 weeks after cell culture establishment), cells were consecutively transduced with the lentiviral particles pRRL-CMV-Ttag-IRES-eGFP and pRRL-CMV-TERT-IRES-CherryFP to obtain partially transformed cells (double transduced; ***D***). Expression of the viral *Ttag* inhibits p53 and retinoblastoma function, and ectopic expression of the *hTERT* gene compensates for proliferation-dependent telomere length attrition. After fluorescent selection by cell sorting, cells were transduced with pLenti-CMV/TORasV12-Puro, which confers a sustained mitogenic signal, and then growth in the presence of antibiotics to select transduced cells (triple transduced; ***T***), which were fully transformed according to Ince and colleagues[6].

As shown in **Figure 4**, a rise in the slope of the regression line (parameter b in the equation 1.2.3) is observed with the increasing number of genetic modifications introduced in BPEC (*N*: 0.47, *D*: 0.93, *T*: 1.13). For a specific interval of time, partially (***D***) and fully transformed (***T***) cells achieved a higher number of population doublings compared to the non-transformed cells (***N***), thus the cell division rate was increased with the transformation process. The same result can also be expressed as the time needed to duplicate the population of cells (t_d), which can be obtained after replacing *y* by 1 (PD) in the equation y = bx, thus $t_d = 1/b$. Contrary to the slope, t_d decreases with transformation. While non-transformed cells needed more than 2 days to duplicate their population (*N*: t_d = 2.13 days), partially transformed cells did it in half the time (*D*: t_d = 1.08 days). The addition of *HRAS* under the regulation of a constitutive promoter led to an increased proliferation activity and cells needed less than 1 day to duplicate (*T*: t_d = 0.89 days) in agreement with the mitogenic activity of this oncogene.

While monolayer cell culture is a useful tool to study in vitro cell behavior, it is a strongly limited

approach because it cannot reproduce most of the physiological conditions. Instead, the three-dimensional cell culture technique described here allows cells from different lineages to aggregate and move freely in a 3D environment forming acinar structures thanks to the cell-cell and cell-matrix junction creation (**Figure 5A**. **Time-lapse**). During the following 2 weeks, cells distribute according to their original tissue function and proliferate increasing the *acini* size (**Figure 5B**). The proper polarization of each *acinus* can be accurately assessed thanks to the combination of immunofluorescent detection of luminal (Claudin-IV) and myoepithelial (Cytokeratin 14) lineage markers with three-dimensional signal location by confocal microscopy (**Figure 6A**). While all the *acini* formed by non-transformed BPECs were properly organized (Claudin-IV positive cells surrounded by Cytokeratin 14 positive cells; **Figure 6Ai**), loss of polarization (**Figure 6Aii**) was observed in *acini* formed by partially and fully transformed BPECs (**Figure 6B**).

One of the main properties of a transformed cell is its ability to grow with the independence of contact with the basal lamina. This property was evaluated by growing the cells embedded on agar for 3 weeks, avoiding its anchorage to plate surface (**Figure 7A**). During the following 3 weeks, those cells with anchorage-independent growth capacity gave rise to colonies composed of multiple cells. As shown in **Figure 8**, 2 days after being seeded in agar, some cells already suffered 2–3 divisions. After 1 week, cells with death-like morphology can be observed indicating that the cells that are not capable of surviving under these conditions eventually died, most likely by *anoikis*. Nonetheless, some cells continue dividing and forming little colonies growing over the second and third weeks of culture.

After adding MTT to the culture, only those cells metabolically active, *i.e.,* alive, are capable to cleave the tetrazolium ring of the MTT resulting in purple MTT formazan crystals 24 hours later (**Figure 7B**). However, these crystals are not only formed by colonies with live cells but also by single cells still alive after 3 weeks in agar (**Figure 9**). Since the purpose of this technique is to determine the ability of cells to proliferate in a substrate-independent manner, the size of the MTT-positive colonies needs to be evaluated. Measurement of colony diameter can be assessed by automatic image analysis (**Figure 7C**) so that small colonies or individualized cells can be filtered. As shown in **Figure 10**, gray dots correspond to those colonies that have suffered less than three divisions in 3 weeks thus measuring less than 65 µm of diameter. These events were included in the data visualization but excluded from the final quantification.

Overall, these results indicate that anchorage independence assay allows discriminating between different degrees of transformation (**Figure 10**). The number of colonies formed by non-transformed BPECs was negligible in comparison to those formed by partially and fully transformed BPECs (colony number: N = 3; D = 278; T = 243). Also, when the colony size was considered, differences between partially and fully transformed states became evident (colony median size: N = 70 µm; D = 83 µm; T = 114 µm). Taking the colony size into account provides more accurate information regarding the tumorigenic potential of studied cell lines and thus it is highly recommended to consider it.

FIGURE AND TABLE LEGENDS:

Figure 1: Screenshot exemplifying image thresholding after MTT staining using Fiji software.

Figure 2: Screenshot of the Extended Particle Analyzer from Biovoxxel plugin conditions and outcome in Fiji software.

Figure 3: Schematic representation of the experimental model of cellular transformation.

Figure 4: Proliferation rate in non-transformed, partially, and fully transformed BPECs. Best-fit lines and 95% confidence bands (dotted lines) of the linear regression are shown. ANCOVA for a linear regression model was applied for the comparison between conditions; * *p*-value < 0.0001. This figure has been adapted from Repullés et al. 2019[7].

Figure 5: *Acini* formation and growth after seeding BPECs in basement membrane matrix. (**A**) Representative images of a time-lapse for the initial times (from 0 to 7 hours) after seeding BPECs in basement membrane matrix. Scale bar = 100 µm. (**B**) *Acini* size over the 14 days of culture in basement membrane matrix for the non-transformed, partially, and fully transformed BPEC. Error bars indicate SEM. No statistical differences between conditions on day 14 (One-way ANOVA and Tukey correction; *p*-value > 0.05). This figure has been adapted from Repullés et al. 2019[7].

Figure 6: *Acini* polarization in non-transformed, partially (*D*), and fully transformed (*T*) BPECs after 3D culture in basement membrane matrix. (**A**) Representative images of a polarized and a non-polarized *acinus* after Cytokeratin 14 (K14, red) and Claudin-IV (Cl-IV, green) immunofluorescence. Polarized *acini* (i) were considered when Cytokeratin 14-positive cells surrounded Claudin-IV positive cells; otherwise, *acini* were considered non-polarized since Cytokeratin 14 and Claudin-IV positive cells were located both in the middle and peripheral locations (ii). Scale bar = 25 µm. (**B**) Percentage of *acini* polarized. A minimum number of 10 *acini* were analyzed for each condition.

Figure 7: Schematic representation of the different steps used for the anchorage-independent growth assay in BPECs. Cells were cultured for 3 weeks in soft agar (**A**) and then MTT staining was applied (**B**). Scale bar = 5 mm. (**C**) MTT positive colonies were quantified using Fiji software. Different steps on image processing are highlighted. Scale bar = 200 µm.

Figure 8: Evolution of cell growth for 3 weeks after seeding individualized cells in 0.3% agar. Images are acquired from a partially transformed BPEC culture. Scale bar = 100 µm.

Figure 9: Representative images of the MTT staining and quantification. Examples with an MTT-positive colony (row 1), two MTT-negative colonies (row 2), and single cells positive or negative for MTT staining (row 3) are shown. A indicates the area of the MTT positive colony or cell. Scale bar = 100 µm.

Figure 10: Colony quantification after anchorage-independent assay in non-transformed (*N*),

partially (*D*), and fully transformed (*T*) BPECs. (**A**) Number and diameter of the colonies obtained for the different conditions. Each dot corresponds to one colony. Soft gray dots represent MTT positive colonies, which were excluded in the results because their diameter was lower than 65 µm (minimum size considered after applying the equation number 4 and taking into consideration at least one division per week). The red line indicates the median colony diameter for each group. Two independent replicas were performed for each condition. Different letters (a, b, c) indicate statistically significant differences for the number of colonies (Fisher's exact test; *p*-value < 0.05) and for their median diameter (Kruskal-Wallis test with multiple comparisons correction; *p*-value < 0.05). This graph has been adapted from Repullés et al. 2019[7]. (**B**) Representative images of entire wells after MTT staining. Scale bar = 5 mm; Inset scale bar = 2.5 mm.

DISCUSSION:

The experimental protocols described in this paper provide useful tools to assess the oncogenic transformation of in vitro cultured cells. Each technique evaluates specific aspects of the transformation process, and thus, special attention must be paid when drawing conclusions from a single analysis. Growth curves build-up is an approach that demands information already available when culturing cells for other purposes. That makes this technique cheaper and easier to apply compared to other cell proliferation assays. However, to obtain valid results, special attention must be paid when counting and seeding cells every time they are sub-cultured. An increased growth rate is indicative of cellular transformation[1], but it should not be used alone because other exogenous factors, such as the addition/removal of antibiotic and antifungal substances or the variation in culture conditions (e.g., temperature, CO_2) can also affect cellular division. It is also important to consider that transduction or the treatment of cells with drugs may affect their growth rate in the upcoming days or weeks and thus give a distorted view of long-term proliferation capacity. In this regard, appropriate controls must be performed.

The 3D culture in basement membrane matrix allows the assessment of cell distribution within a whole functional entity, the *acinus*. An altered organization is indicative of an intercellular communication impairment that could lead to a loss of function, a characteristic of tumoral tissues. The fact that some *acini* present non-polarized organization indicates that some cells have initiated the transformation process. Regarding technical issues, it is important to accurately determine the optimal concentration of seeded cells and the concentration of the matrix. These two parameters can influence the number and size of the resulting *acini* and this could interfere with their organizational capacity. Also, manipulation of basement membrane matrix requires a certain degree of experience as it must be gently handled. Despite being a laborious technique, the growth of cells in three dimensions resembles the physiological context of these cells and allows the evaluation not only of the distribution of breast lineage markers[7, 15] but also of other structures that provide information about tumoral features, such as the disruption of the basement membrane[16]. 3D cell cultures represent the future in cell culture research. In fact, 3D growths can give rise to more physiological and interesting findings while taking into account microenvironment elements and providing us with a lot of different studies as well as therapeutical targets' identification and evaluation, cell to cell interactions, or stem cell investigations.

Anchorage-independent growth of adherent cells is an unequivocal treat of the transformation process. While some of the partially and fully transformed BPECs were still able to give rise to a structured *acinus*, they manifest their ability to form colonies when forced to grow individually in suspension. At the technical level, the anchorage assay is also complex since small variations during agar manipulation (e.g., high temperature) or during disaggregation of cells after trypsinization can notoriously affect cell colony formation. However, the material required is cheaper than basement membrane matrix used for 3D cell cultures making the assessment of the anchorage-independent growth more affordable. Also, prior to MTT addition, single colonies can be picked and allowed to grow out of the agar in plates with an adherent surface. These colonies may result in clonal cell lines that may continue growing in suspension or adhere again. DNA, RNA, and/or protein can be extracted from these cell cultures for further analyses.

There is a general limitation regarding the methods described here: they are very time consuming, and it takes several weeks to obtain the results. However, since each test assesses a specific tumor characteristic, conclusions made considering the whole set of results are very sound. Therefore, all the three tests together are powerful indicators of cellular transformation.

ACKNOWLEDGMENTS:

The AG laboratory is funded by the Spanish Nuclear Safety Council. T.A. and A.G. are members of a research group recognized by *Generalitat de Catalunya* (2017-SGR-503). MT holds a contract funded by the Scientific Foundation *Asociación Española Contra el Cáncer* [AECC-INVES19022TERR]. G.F. contract is funded by a grant from Cellex Foundation.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

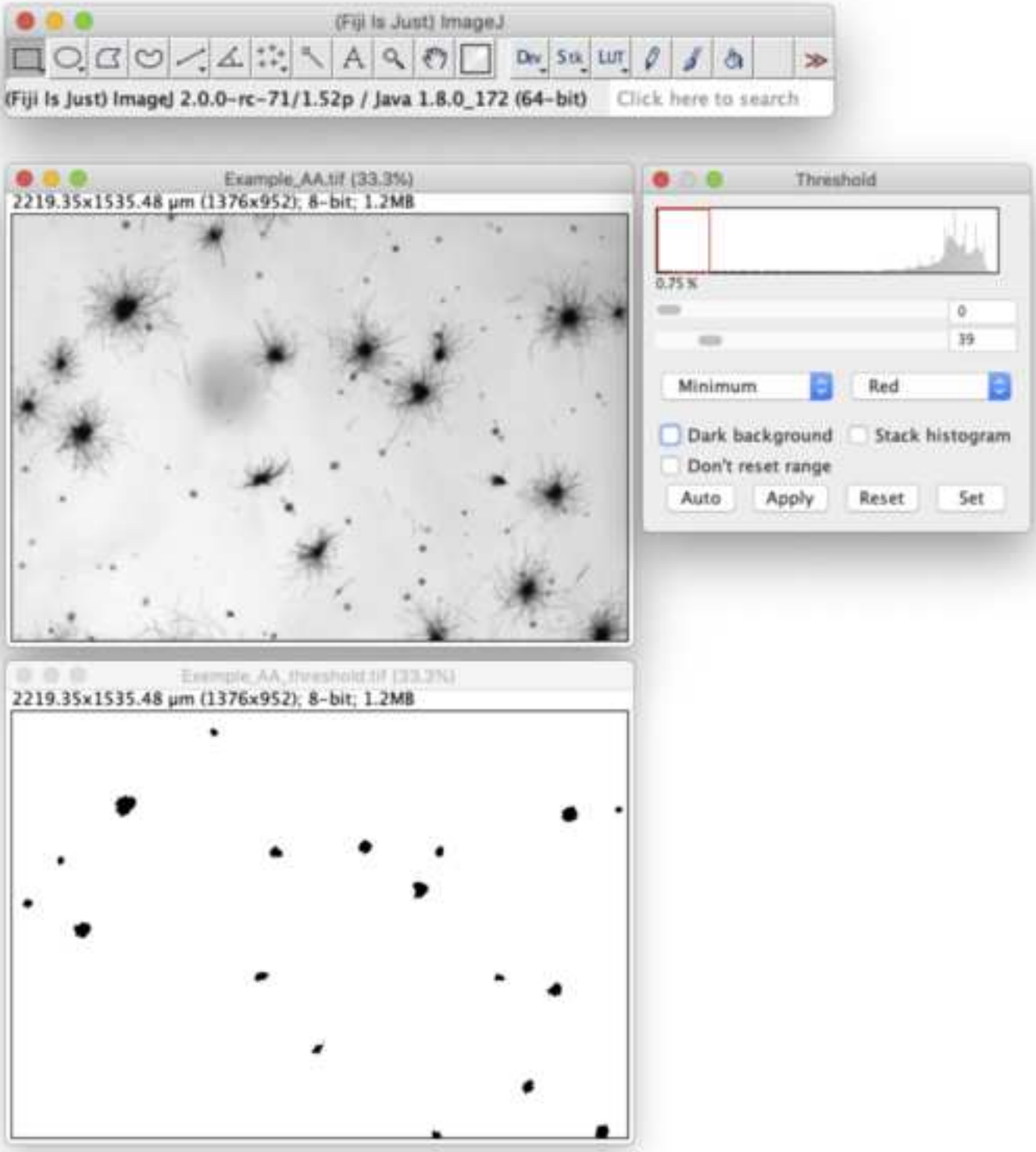


Figure 2

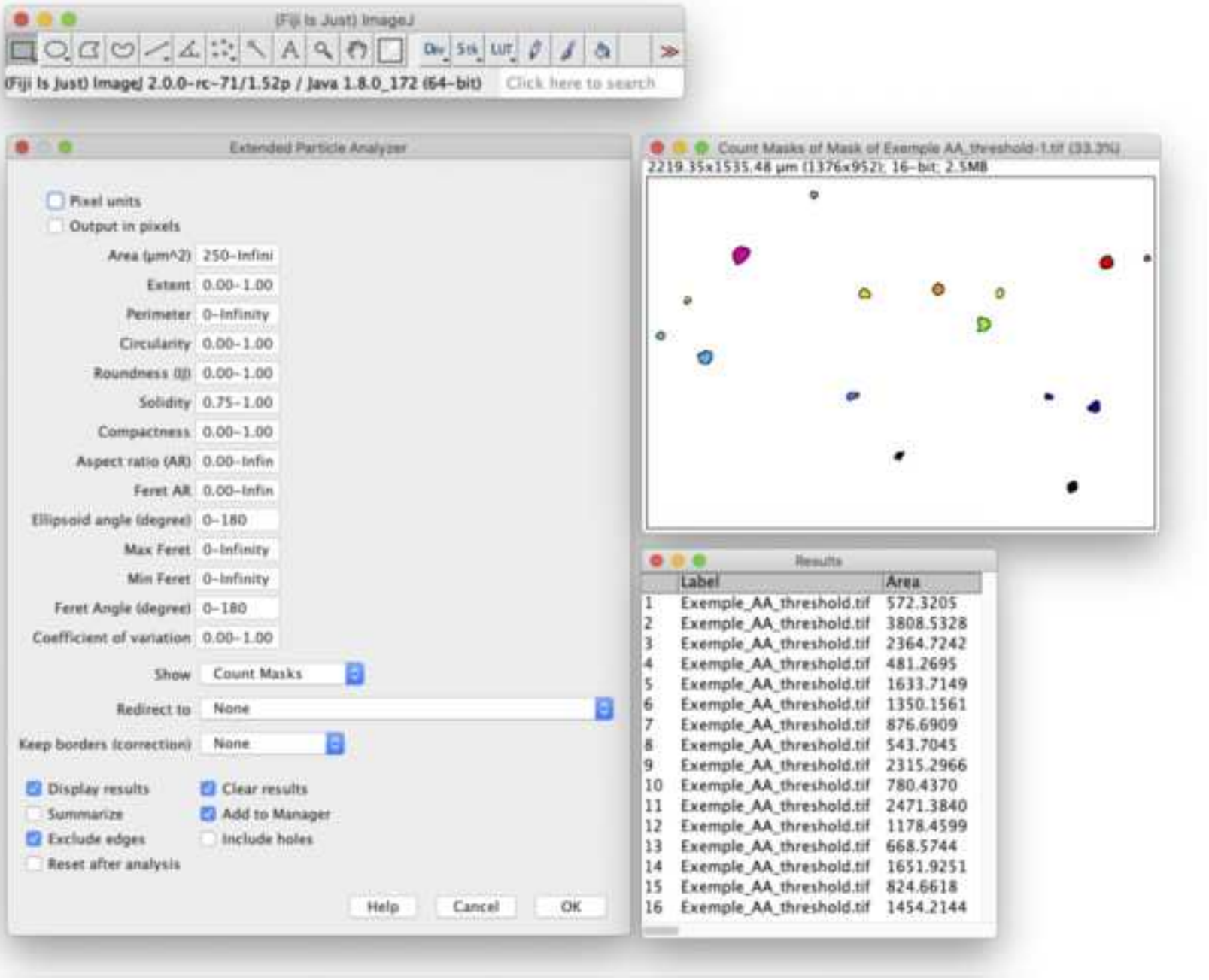


Figure 3

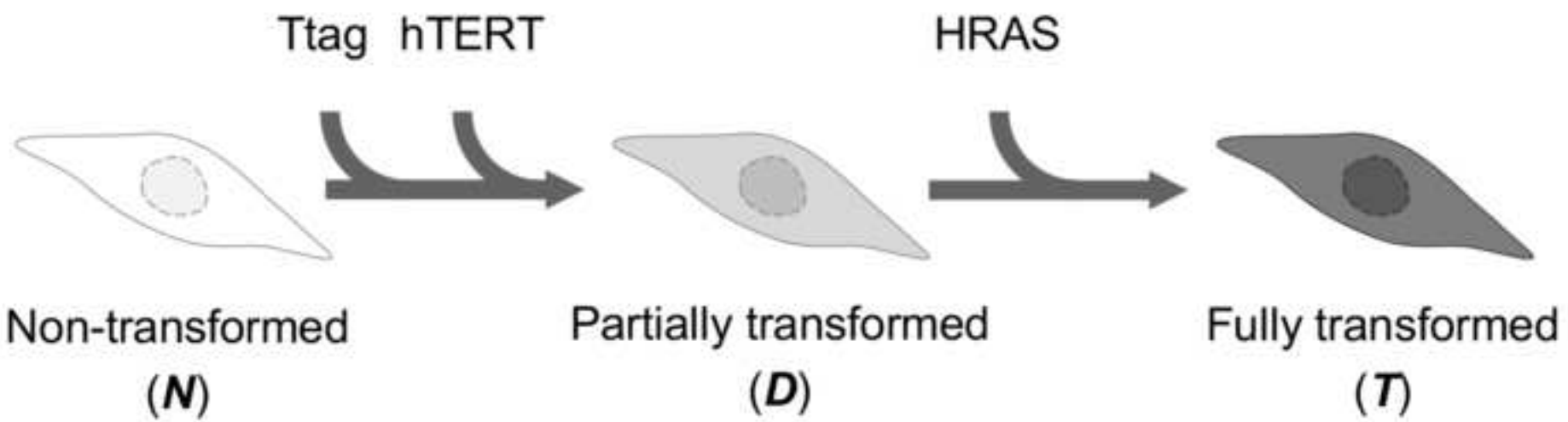


Figure 4

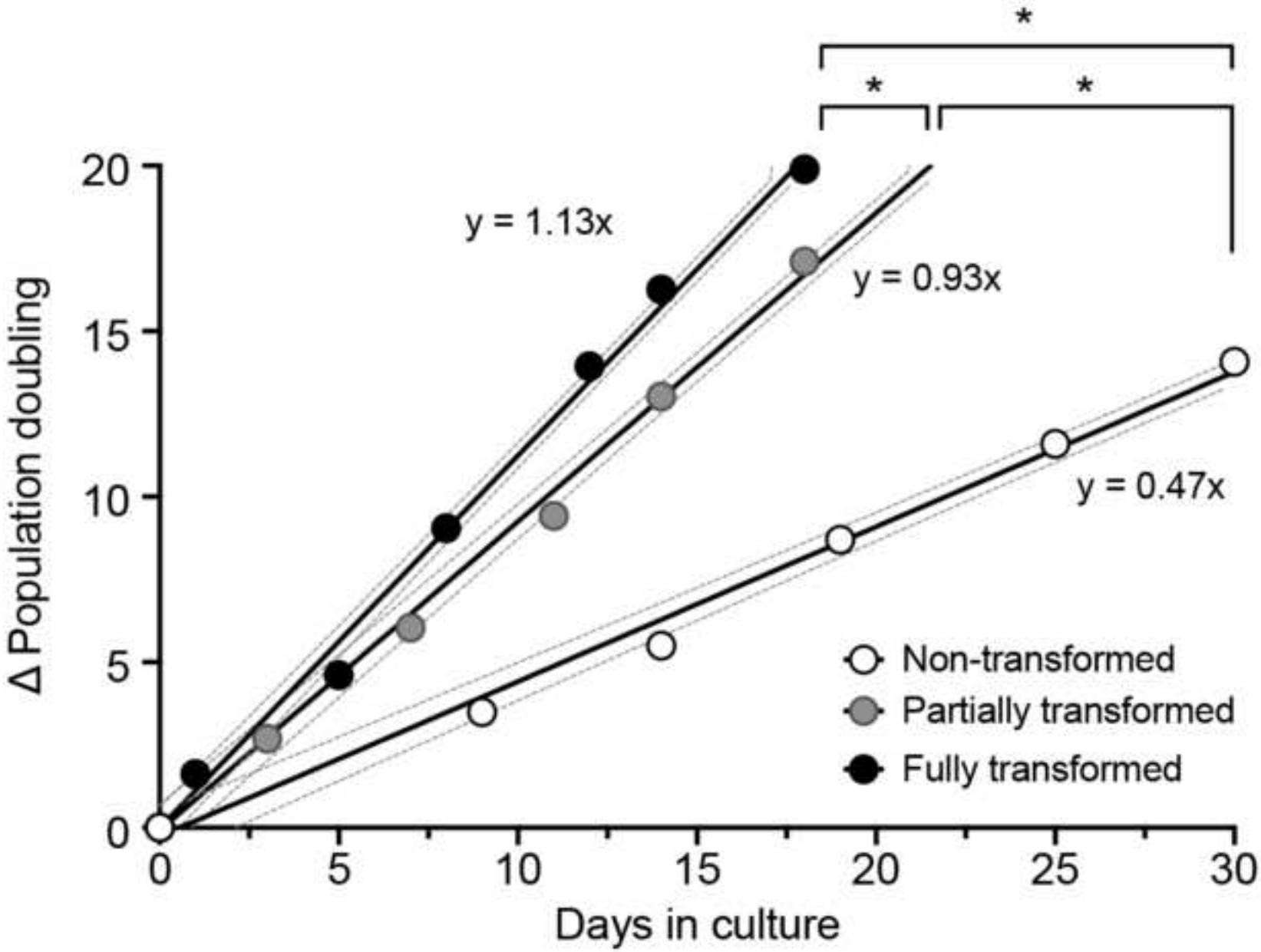


Figure 5

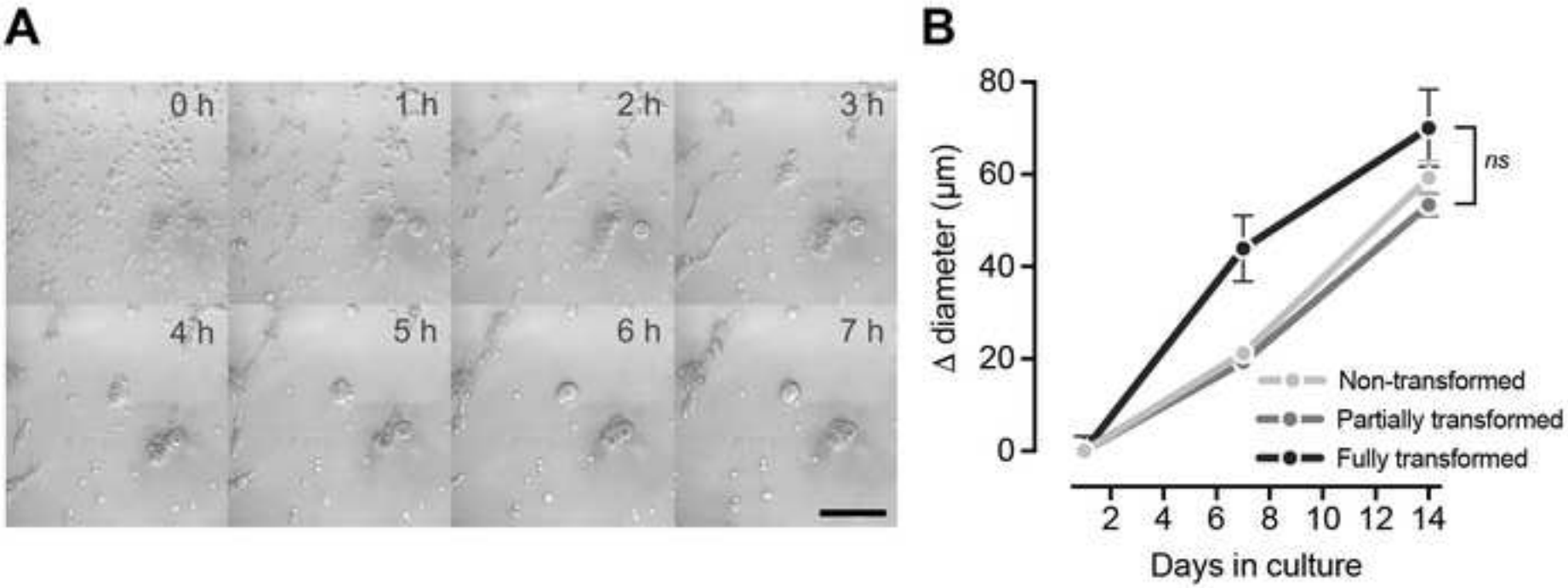


Figure 6

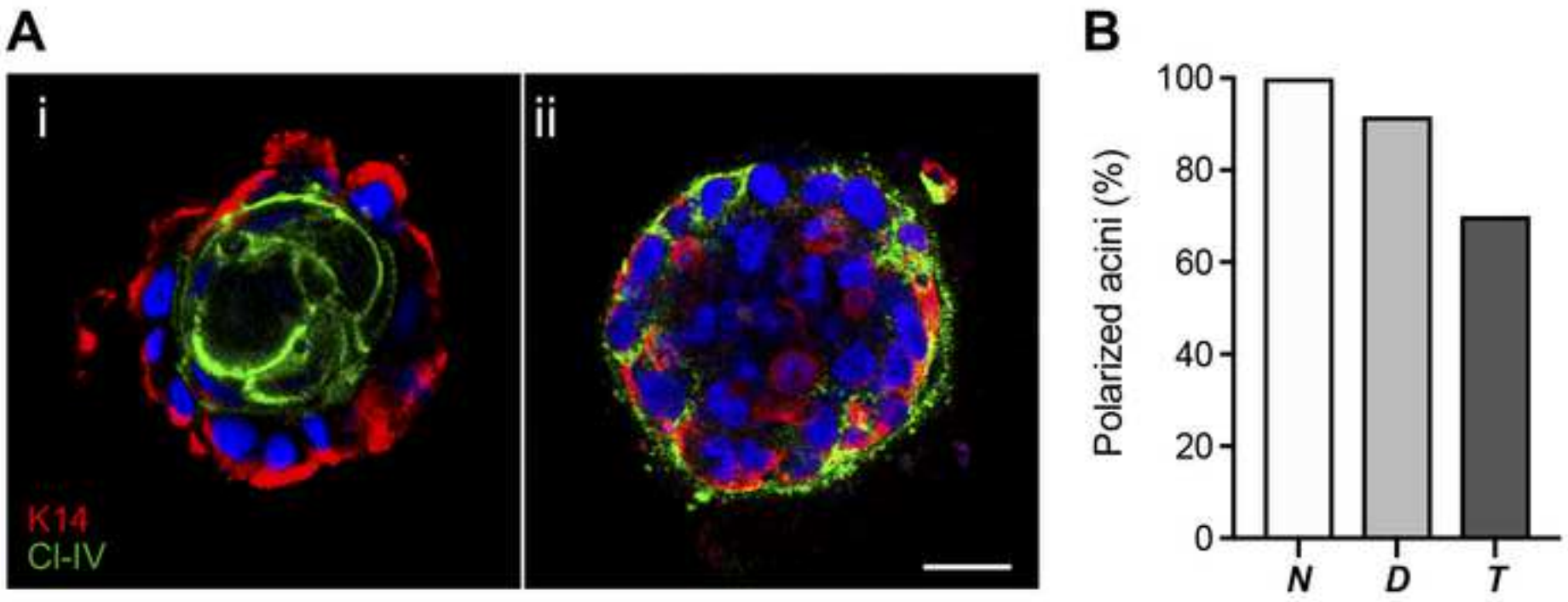


Figure 7

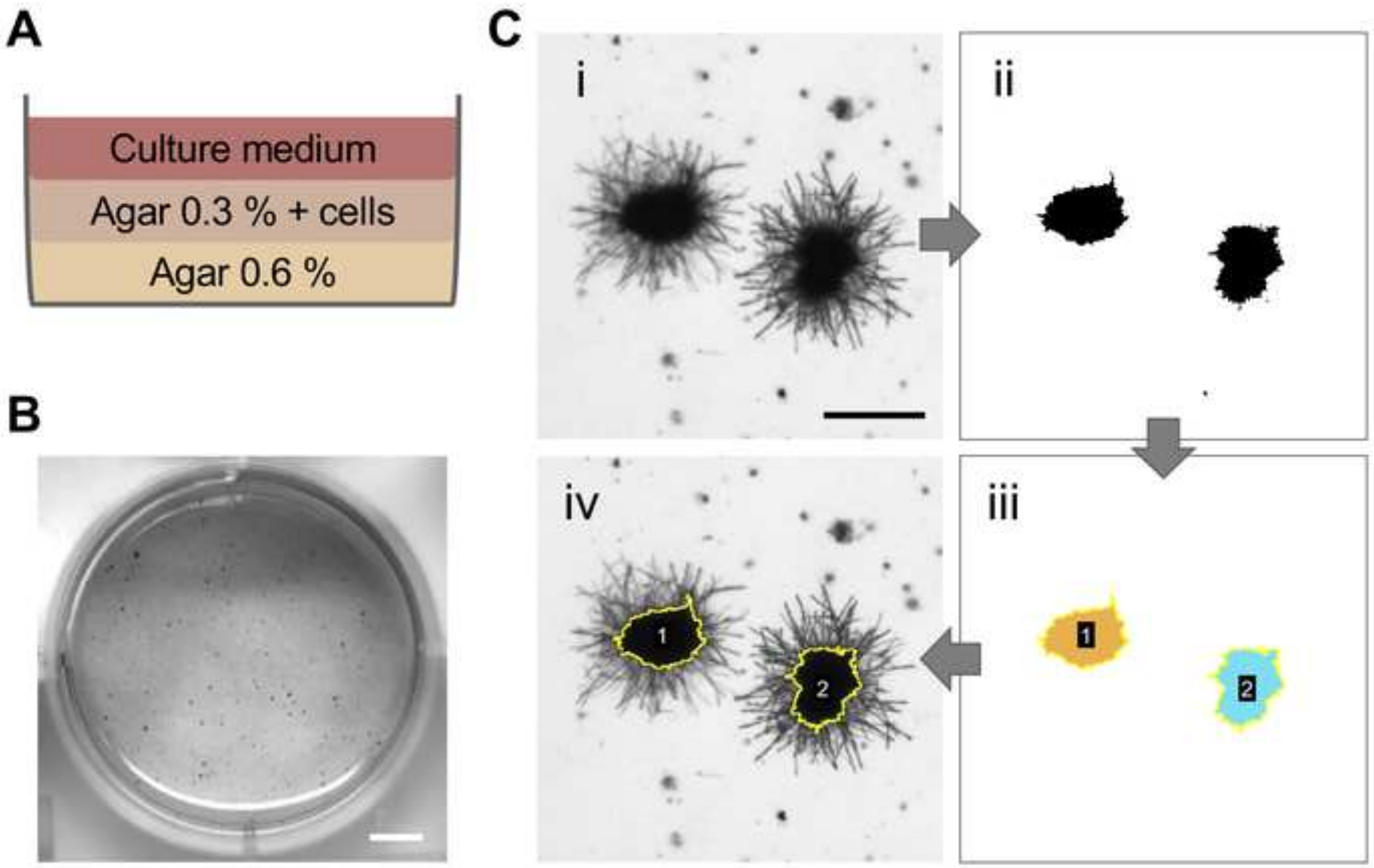


Figure 8

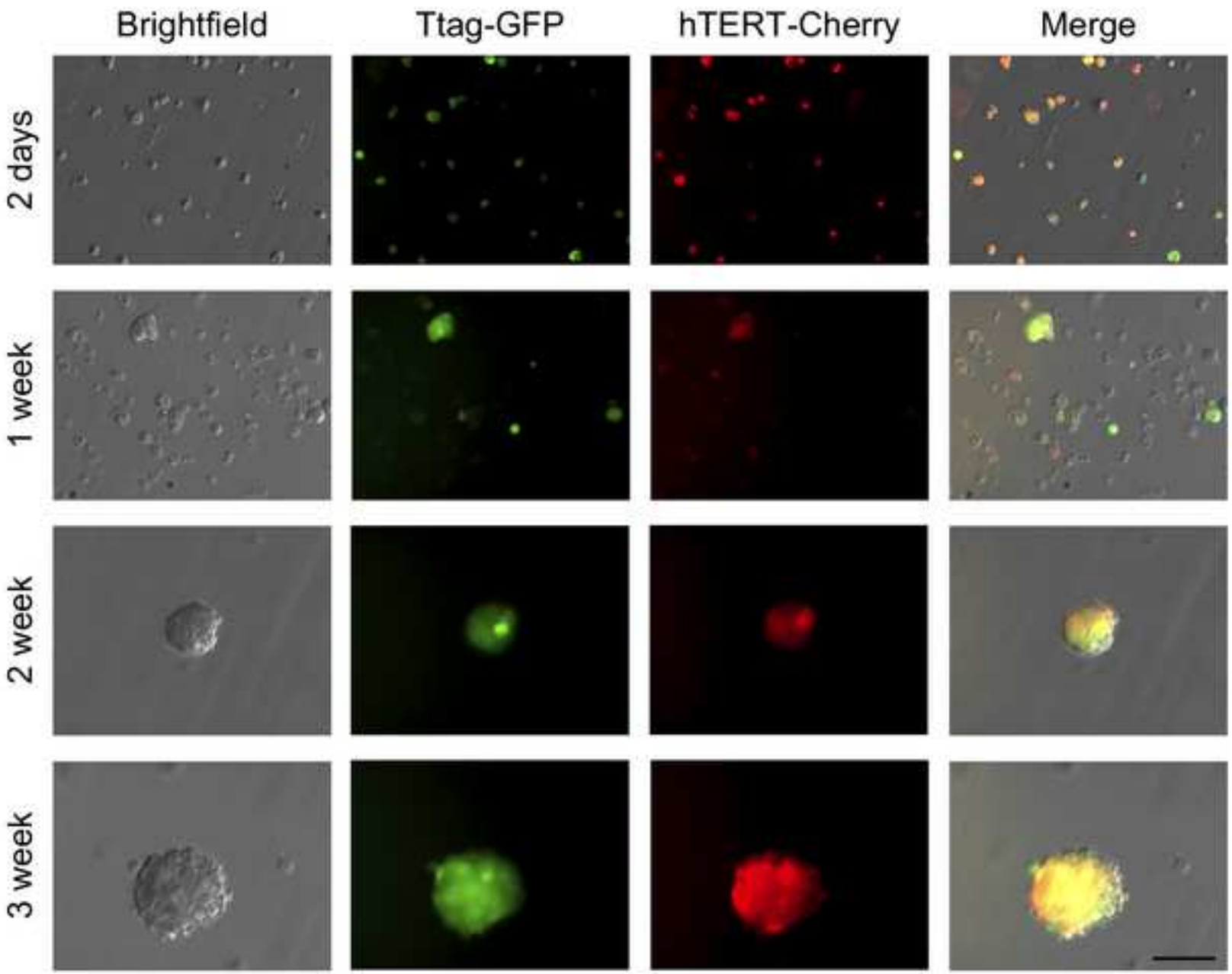


Figure 9

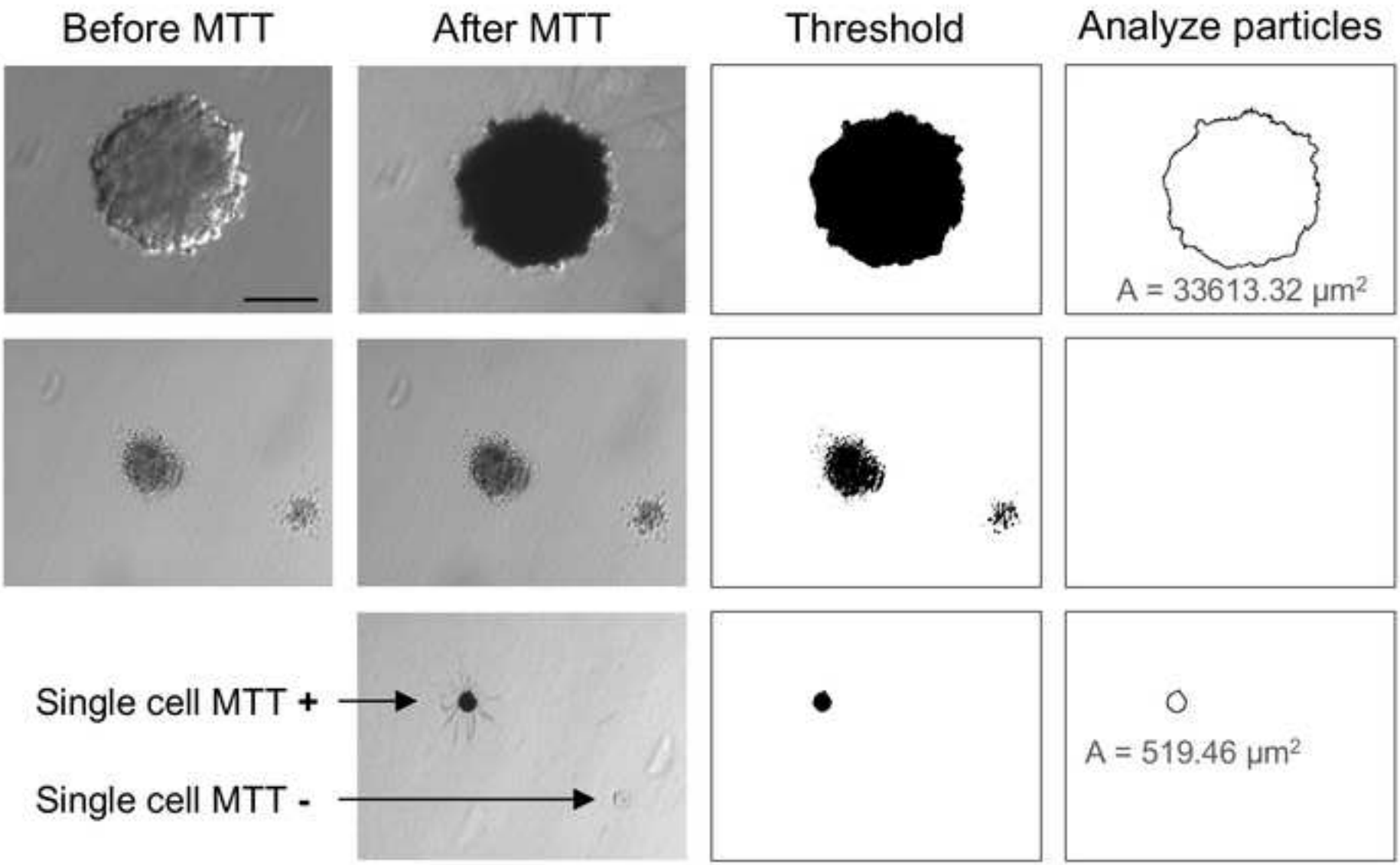
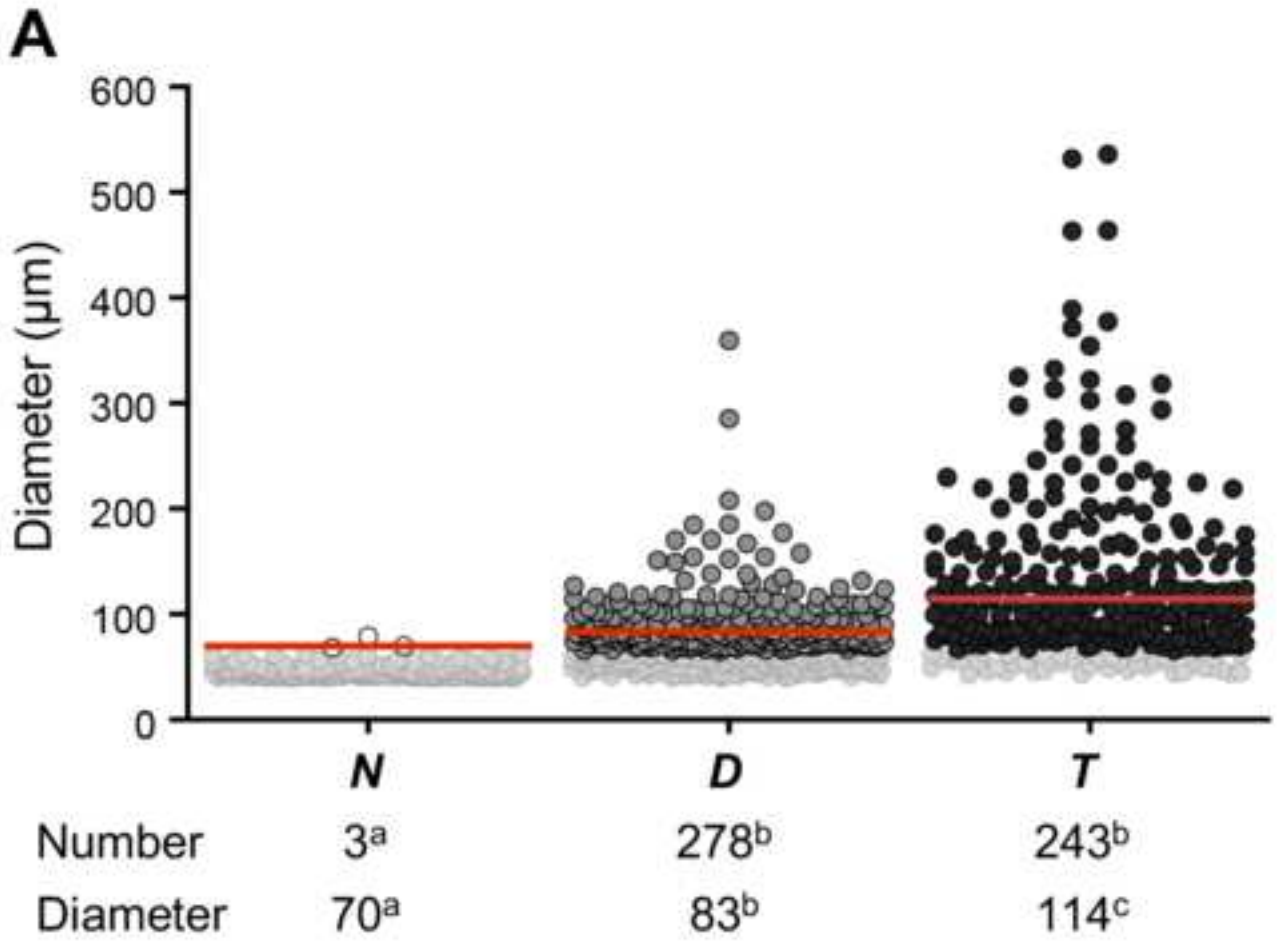


Figure 10



Name of Material/Equipment	Company	Catalog Number
1 ml Serological Pipettes	Labclinics	PLC91001
1.5 ml Eppendorfs	Thermo Fisher Scientific	3451
10 µl Pipette tips w/o filter	Biologix	20-0010
100 ml glass bottle		
1000 µl Pipette tips w/ filter	Labclinics	LAB1000ULFNL
1000 µl Pipette tips w/o filter	Biologix	20-1000
15 ml Conical tubes	VWR	525-0400
2 ml Serological Pipettes	Labclinics	PLC91002
200 µl Pipette tips w/ filter	Labclinics	FTR200-96
5 ml Serological Pipettes	Labclinics	PLC91005
50 ml Conical Tubes	VWR	525-0304
Acetone	PanReac AppliChem	211007
Agar	Sigma-Aldrich	A1296
Anti-Claudin 4 antibody	Abcam	15104, RRID:AB_301650
Anti-Cytokeratin 14 [RCK107] antibody	Abcam	9220, RRID:AB_307087
Anti-mouse Cyanine Cy3 antibody	Jackson ImmunoResearch In	115-165-146, RRID:AB_233869
Anti-rabbit Alexa Fluor 488 antibody	Thermo Fisher Scientific	A-11034, RRID:AB_2576217
Autoclave		
BioVoxxel Toolbox		RRID:SCR_015825
Cell culture 24-well Plate	Labclinics	PLC30024
Cell culture 6-well Plate	Labclinics	PLC30006
Cell incubator (37 ºC and 5 % CO2)		
Cell Strainers	Fisherbrand	11587522
CellSense software	Olympus	
Centrifuge		
Cholera Toxin from Vibrio cholerae	Sigma-Aldrich	C8052
Class II Biological Safety Cabinet	Herasafe	HAEREUS HS12
Confocal inverted Microscope	Leica	TCS SP5
Cover glasses	Witeg Labortechnik GmbH	4600122
DAPI		
Fetal Bovine Serum	Biowest	S1810
Fiji software (ImageJ)	National Institutes of Health	RRID:SCR_002285

Glass Pasteur Pipettes		
Glass slides	Fisherbrand	11844782
Goat Serum	Biowest	S2000
Heat-Resistant Gloves		
Heater bath (37 ºC)		
Heater bath (42 ºC)		
Heating plate		
Humid chamber		
Ice		
Ice-box		
Inverted Optic Microscope	Olympus	IX71
Matrigel Matrix	Becton Dickinson	354234
Methanol	PanReac AppliChem	131091
Micropipette		
Microsoft Office Excel	Microsoft	RRID:SCR_016137
MilliQ water		
Nail Polish		
Parafilm M	Bemis	PM-999
PBS pH 7.4 (w/o calcium & magnesium)	Gibco	10010-056
PBS tablets	Sigma-Aldrich	P4417
Pipette Aid		
Primaria T25 flasks	Corning	353808
Scepter Automated Cell Counter	Millipore	PHCC20060
Scissors		
Sterile filters 0.22 µm	Millipore	SLGP033RS
Thiazolyl Blue Tetrazolium Bromide (MT	Sigma-Aldrich	M2128
Triton X-100	Sigma-Aldrich	T8787
Trypsin-EDTA 10X	Biowest	X0930
Vectashield Antifade Mounting Medium	Vector Laboratories	H-1000
WIT-P-NC Culture Medium	Stemgent	00-0051
WIT-T Culture Medium	Stemgent	00-0047

	Comments/Description
	Dark eppendorfs are preferred for MTT long-term storage
	With cap, autoclavable
	Used for 3D structure fixation prior to immunofluorescent labelling Used for anchorage assay Working dilution 1:100, host: rabbit Working dilution 1:100, host: mouse Working dilution 1:500, host: goat Working dilution 1:500, host: goat
	Used for 3D cultures in Matrigel. Flat Bottom Used for anchorage assay
	Mesh size: 40 µm Used to image acquisition
	Used to supplement cell culture medium
	22 X 22 mm, thickness 0.13 - 0.17 mm 2-(4-amidinophenyl)-1H -indole-6-carboxamidine Used to inactivate trypsine action Free download, no license needed

Used for immunofluorescence of 3D structures
Used for agar manipulation after autoclave
Used to temper solutions prior to cell subculture
Used to keep agar warm
Used for Matrigel dehydration
Used for the incubation of antibodies during immunofluorescence
Used during Matrigel manipulation

Store at -20 ºC and keep cold when in use. Referred to as basement membrane matrix
Used for 3D structure fixation prior to immunofluorescent labelling
p1000, p200 and p10
Used to calculate population doubling and to obtain growth rate equation
Referred to as ultrapure water
Used to seal samples after mounting
Used to cover antibody solution during incubation
Sterile. Used for cell subculture
Dilute in milliQ water. No sterility required. Used for immunofluorescence

Used for BPEC culture
Alternatively, use an haemocytometer
Used to cut pipette tips and parafilm
Used to filter MTT solution
Store at -20 ºC
Used for immunofluorescence of 3D structures
Dilute in PBS to obtain 3X solution

Used for primary BPEC culture
Used for transformed BPEC culture

Dr. Vineeta Bajaj
Review Editor
Journal of Visualized Experiments

Dear Dr. Bajaj,

Our manuscrit, JoVE 61716 entitled "Techniques for the *in vitro* evaluation of oncogenic transformation in human mammary epithelial cells" has been revised according the reviewers' and the editor comments and our submission has been uploaded. Together with our submission, we have also uploaded a separate letter that addresses each of the editorial and peer review comments individually.

We look forward to your reply,

Yours sincerely,
Teresa Anglada and Anna Genescà

Response Letter to the Editorial and the Reviewers

We are thankful for the deep revision of the manuscript by the Editorial, and we have thoroughly revised the concerns addressed. We also appreciate the positive feedback from the reviewers. The followings are our point-by-point responses to the editorial and reviewer's comments:

Editorial Comments

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Following the editorial advice, we have thoroughly checked grammar and spelling and changes applied can now be tracked on the edited version of the manuscript.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We understand the editor's concerns thus we have ensured that all the steps included in the protocol can be filmed and, most importantly, replicated by other researchers.

1) 2.1.6: mention centrifuge speed and duration.

After trypsinization cells are centrifuged at 500 x *g* for 5 minutes. We omitted this information in the previous version because it was already explained in 1.1.2.9. However, we have now included the centrifuge speed and duration in 2.1.6. in order to avoid confusion.

2) 2.2.9, 2.2.11: mention antibodies and their concentrations

We have added a note in 2.2.8. with detailed information regarding primary and secondary antibodies description and recommended working concentration. "NOTE: Antibody concentration must be accurately adjusted depending on the cell type and the antibody reference. As a guide, for BPECs use primary anti-Cytokeratin 14 and anti-Claudin-IV antibodies (see Table of Materials) at a working solution concentration 1:100 and secondary anti-Mouse and anti-Rabbit antibodies (see Table of Materials) at 1:500 working solution concentration."

3) 2.2.15: Provide microscope magnification, fluorescence excitation and emission filter settings etc.

Following the editorial advice, we have included the microscope configuration parameters in 2.2.15.

"NOTE: Confocal microscope configuration must be accurately determined depending on the equipment used and the antibodies applied to the sample. As a guide, with the equipment and reagents detailed in the Table of Materials use a 40x objective and the following laser and detector settings:

- DAPI: excitation with a 405 laser (5%), detection with a PMT detector (800V, Offset: -10) and a spectral band from 410 nm to 500 nm.
- A488 (Claudin-IV): excitation with a 488 laser (10%), detection with a PMT detector (800V, Offset: -20) and a spectral band from 490 nm to 550 nm.
- Cy3 (Cytokeratin 14): excitation with a 555 laser (10%), detection with a PMT detector (800V, Offset: -35) and a spectral band from 560 nm to 600 nm."

• **Protocol Highlight:**

1) 1.2 is not filmable, please unhighlight.

We think that step 1.2. is essential for a complete understanding of the methodology. If we omit this part, the protocol regarding the obtention of the number of cells after subculture (1.1.) loses its sense. When we decided to highlight this part we thought that we could record our screen while doing the calculations of the accumulated population doubling. We have now reduced the highlighted length but we are wondering if it is possible to reconsider to include part of step 1.2. in the video.

2) 3.3.3, 3.3.4: unhighlight these due to low filmable content.

According to the editorial comment steps 3.3.3, 3.3.4 have been unhighlighted.

3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

Thanks for the comment, we have ensured that the highlighted steps follow a cohesive narrative. To do so, we have highlighted/unhighlighted some words or sentences and changes applied can now be tracked on the edited version of the manuscript.

4) Please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

According to the editorial comment, we have re-evaluated the length of the protocol section and we consider that all steps included now are necessary for the reproduction of the protocol by other researchers. We have made sure that the protocol section does not exceed the length limit (10 pages maximum). Regarding the highlighted text, we have verified that it is no longer than recommended.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Since we provide a set of tools to evaluate the oncogenic transformation of cells, we thought the best way to discuss the methodology presented was to do it tool by tool. Thus, we addressed all points required to be covered in a single paragraph for each tool. If a researcher only wants to use 1 or 2 of the tools presented it is easier to follow up all the discussion for this specific tool continuously and not have to jump from one paragraph to the next. We have added an extra paragraph to discuss some points in a more transversal way across the different tools.

Here we provide an example of a paragraph discussing the 3D technique with the required topics highlighted following a color code:
1) modifications and troubleshooting,
2) limitations of the technique,
3) significance with respect to existing methods,
4) future applications and
5) critical steps within the protocol.

[...]
The 3D culture in basement membrane matrix allows the assessment of cell distribution within a whole functional entity, the acinus. An altered organization is indicative of an intercellular communication impairment that could lead to a loss of function, a characteristic of tumoral tissues. The fact that some acini present non-polarized organization indicates that some cells have initiated the transformation process. Regarding technical issues, it is important to accurately determine the optimal concentration of seeded cells and the concentration of the matrix. These two parameters can influence the number and size of the resulting acini and this could interfere their organizational capacity. Also, manipulation of basement membrane matrix requires a certain degree of experience as it must be gently handled. Despite being a laborious technique, the growth of cells in 3 dimensions resembles the physiological context of these cells and allows the evaluation not only of the distribution of breast lineage markers (LaBarge et al., 2013 JOVE; Repullés et al., 2019) but also of other structures that provide information about tumoral features, such as the disruption of the basement membrane (Zubeldia-Plazaola et al. 2018). 3D cell cultures represent the future in cell culture research. In fact, 3D growths can give rise to more physiological and interesting findings, taking into account microenvironment elements and providing us with a lot of different studies as well as therapeutical targets identification and evaluation, cell to cell interactions or stem cell investigations.
[...]

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Primaria, Matrigel, Vectashield, MilliQ,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
We have replaced the commercial language in our manuscript for generic names. Changes applied are detailed below and can be tracked on the edited version of the manuscript.
· Primaria → modified surface
· Matrigel → basement membrane matrix
· Vectashield → antifade mounting medium
· MilliQ → ultrapure water
· Parafilm → laboratory wrapping film

• **Table of Materials:**

1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as antibodies and RRIDs,

We have revised the Table of Materials and Equipment and we have confirmed that it includes all the essentials. We have added the company and reference number to a couple of reagents where it was missing. However, in some cases, it cannot be provided because we have done it ourselves (e.g. humid chamber) or it is of general usage (e.g. Pipette Aid, heat resistant gloves). For other products, we have detailed the properties required on a comments/description column (e.g. 100 ml glass bottle: with cap, autoclavable). Antibodies used were already listed, however, we have now included the word “antibody” in their description to make them more easily found. RRIDs have been added where applicable.

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Registered trademark symbols have been removed from the Table of Materials.

3) Please sort in alphabetical order.

Done

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Please, find attached a supplemental file with the permission request. Where applicable, the citation is included in the figure legend.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Although the in vitro transformation assay represents a historical approach to study the oncogenic transformation, it still retains some values in cancer research. Hence, the protocol here presented is of some interest. Enclosed my specific points

PDi Population doubling of the seeded cells. It should be better explained how to get it.

We thank the reviewer for this suggestion as we agree that a better explanation of this parameter was convenient. The variable PDi refers to the number of Populations Doublings accumulated by the cells on the previous counting made. Since PD is the accumulated population doubling for a specific time point, the number of population doublings that these cells already achieved must be taken into consideration.

We have clarified this issue in the manuscript as follows: "PD_i: Number of population doublings achieved by the cells until the previous subculture (it refers to the PD accumulated on the previous subculture)"

I am wondering whether fixation with methanol:acetone (1:1, v/v) is the only options?

We thank the reviewer for raising this issue since, as a general rule, the fixative for immunofluorescent labeling depends on the target epitope. However, when working with Matrigel attention must be paid to the fixation step because some fixatives, as PFA, may cause de-polymerization of the matrix and the consequent loss of 3D structures on the following washing steps. The addition to 1% glutaraldehyde helps to solve the de-polymerization problem when using PFA, although it may produce background fluorescence. From our experience, better results are obtained with methanol:acetone in comparison to PFA for all the antibodies we have tested.

Besides, in our experiments, cells used for acini formation were previously transduced with exogenous genes along with reporter genes encoding for fluorescent proteins (mCherry and EGFP) to be able to select transduced cells by fluorescence-activated cell sorting. Thus, the removal of intrinsic fluorescence was crucial to then perform immunofluorescent labeling of the proteins of interest. This was achieved with the fixation of the samples with methanol:acetone, as commented on the NOTE of 2.2.5.

In figure 6A transformed acini show a sort of inverted polarization. Any comments?

We understand that the first impression may be of an inverted pattern of polarization. However, despite in the non-polarized acinus of figure 6A most of the cells with CI-IV (green) occupy a peripheral location, some K14 (red) positive cells remain on the periphery. This picture was chosen because it shows a very good example of a non-polarized acinus, which is characterized by the high number of CI-IV (green) cells capable to surpass the K14 (red) peripheral belt. However, it's not always so evident. Acini can be classified as no-polarized even when a single CI-IV positive cell is placed on the periphery of the acinus because it is indicative that the regulation of cellular localization has been lost. Here we provide another example of a non-polarized acinus where there is a cell in the 3rd stack in the central position that is clearly CI-IV (green) positive. Also, in the following slices, there are some K14 (red) positive cells in the periphery. We have also added a note in the figure legend of figure 6A to clarify this issue.

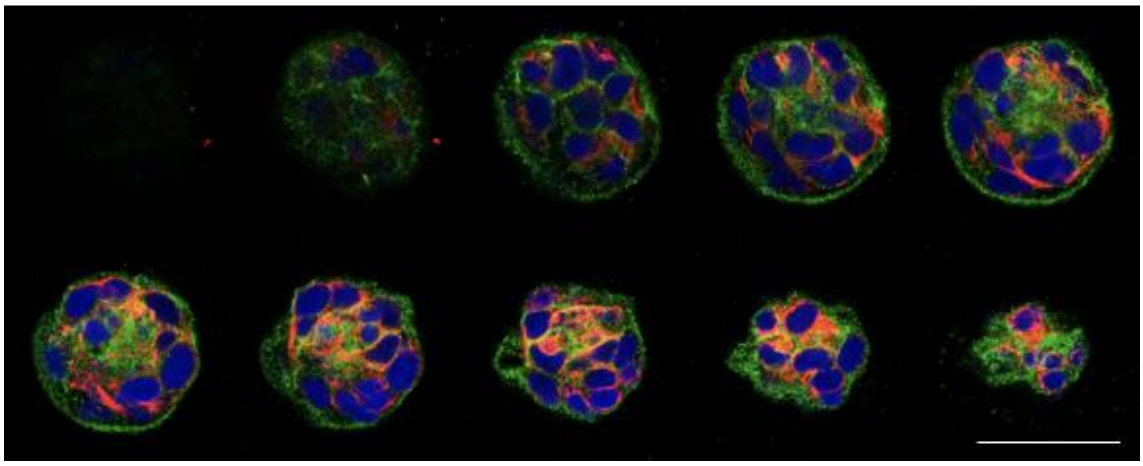


Figure 1. Example of a non-polarized acinus. Each image corresponds to a z-stack. Positive cells for CI-IV (green) and K14 (red) are located both in middle and peripheral positions. Scale bar: 50 μm.

Figure 6B shows that the vast majority of transformed cells still retain polarized acini. Any comments?

We thank the reviewer for raising this point. Matrigel provides a 3D environment that allows cells to survive, grow and move freely. Compared to soft-agar assay, the culture of cells in Matrigel keeps them in less strict conditions and allows the proliferation, not only of those cells capable to form colonies in suspension, but of all of them.

It is known that, only a subset of cells are able to start a neoplastic process. In this experimental approach, even if cells from both lineages (luminal and myoepithelial) have been transduced, only some of them may have acquired mutations providing high fitness and thus being able to give rise to a tumor. Moreover, it is important to note that acini are formed from the aggregation of different cells, and even if a cell is capable to form a tumor, more time should be needed for a unique cell to affect the polarization of a whole acini which is mainly formed by cells with less fitness. Therefore, from our point of view, it is not surprising that the majority of cells present in acini are still capable of forming organized structures. Taking this into account, it is worth noting that we still observe a reduction of nearly 35% of polarized acini compared to their non-transduced counterparts.

The image in figure 7B is too dark and of low quality. Please, improve it. A color image could help.

The reviewer is right and the brightness and contrast have been adjusted. We believe a color image will not improve the quality of the image since colonies after MTT staining are dark. We hope such images give researchers an idea of how colonies are seen by the naked eye.

The same point for figure 10B.

The same approach has been followed. We hope the inset in these images provides an idea of the colonies' magnitude depending on the condition analyzed.

The question of discriminating between partially transformed and fully transformed cells is important.

We completely agree with the reviewer. The analysis of colony size offers valuable information to obtain conclusions on the degree of cellular transformation. As shown in the Results section (figure 10A), taking into consideration the number of colonies alone is not always enough to discriminate intermediate stages of cellular transformation (D = 278 colonies vs T = 243 colonies). However, the information on the colony size makes it possible to differentiate them. More details regarding this topic can be found in the last paragraph of the Representative Results section.

Can they resolve this point through a detailed analysis of colonies dimensions? Yes, the mean diameter of D is 83 µm while for T is 114 µm, which is significantly bigger. Not only the average diameter of all colonies but also the presence of colonies with much bigger dimensions are indicative of a higher degree of transformation.

Can they identify a percentage of increasing (surface) between the two conditions?
Considering the average diameter of colonies for the two conditions the surface can be calculated as follows:

Data:

Mean diameter for **D: 83 µm**

Mean diameter for **T: 114 µm**

Calculation considering 2D colonies:

$Area = \pi \cdot r^2$

Area D: 5,410.61 μm^2

Area T: 10,207.03 μm^2

There is an increase of 4,796 μm^2 on average for T compared to D, which means that T have almost doubled the surface of the D.

The same approach can be followed to calculate surfaces in 3D dimensions.

Calculation considering 3D colonies:

$Volume = 4/3 \cdot \pi \cdot r^3$

Volume D: 299,386.97 μm^3

Volume T: 775,734.62 µm

There is an increase of 476,347.65 μm^3 on average for T compared to D, which means that T have more than doubled the volume of the D.

Frequently as they show in figure 10A there is a certain degree of heterogeneity in soft-agar foci dimension. Can they comment this point?

The reviewer's observation is interesting. Heterogeneity comes from the fact that the agar prevents the free movement of cells, making it difficult to form large colonies and preventing the fusion of different colonies. In a fully transformed cell line, some of the colonies can be small but what is important is their capacity to form some colonies of bigger dimensions. In order to detect these bigger colonies it is important to score a large number of colonies and for this reason we recommend to score the colonies formed all

over the plate. Also, in order to draw conclusions from agar experiments we recommend taking into account both the number and size of the colonies.

In the soft agar assay branched cells emerge from the border of the foci. Can the authors comment this point?
We are not sure to understand the point of the reviewer since we did not observe branched cells emerging from colonies in our experiments. If the reviewer refers to filaments that protrude from the main body of the colony once MTT applied we want to make clear that it often appears as a result of converting MTT to crystal formazan. For example, in figure 2 the area inside the yellow line corresponds to a colony of cells metabolically active. The branches outside this yellow area appear as a result of crystallization and are not considered in the colony size measurement.

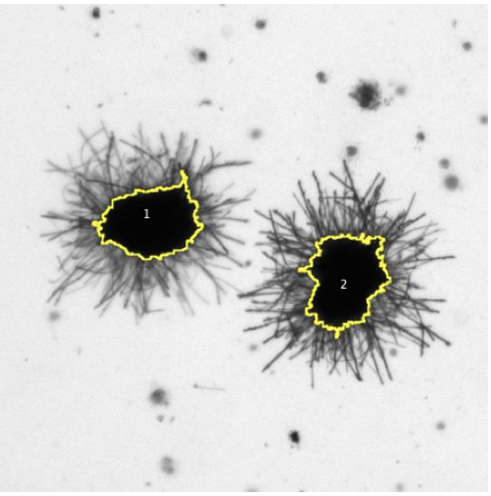


Figure 2. Representative image of MTT-positive colonies.

Reviewer #2:
Manuscript Summary:
Genesca et al. Has described a method to evaluate the transformation of normal cells into oncogenic cells after genetic manipulation. These tools include both cellular assays and image analysis.

Minor Concerns:
1) In 2.1 please mention what kind of plates have been used for matrigel experiment. The amount of matrigel matters to type of plates used.
We thank the reviewer for the comment. We have moved the type of plates used (24 well plates) from step 2.1.1. in the protocol section to a NOTE in 2.1 to make it easy for the readers to find this information. We have also indicated with a comment in the Table of Materials that the plates must have a flat bottom.

2) Instead of MTT assay, have you evaluate using crystal violet? IS there a difference in calculating colonies?
While MTT assay measures the metabolic activity of cells as an indicator of cell viability, crystal violet assay is not able to distinguish live and dead cells. Viable cells with an active metabolism can reduce the yellow tetrazolium salt (MTT) to purple formazan crystals that are microscopically visible. Otherwise, death cells lose this ability and crystals are not formed. In contrast, the crystal violet is a triarylmethane dye with an

affinity for DNA. This assay assumes that cells attached to the plate are alive and that dead cells have been detached. Thus, although crystal violet is recommended for assays with adherent cells it is not a good option for agar assays because death cells will continue embedded in the agar. That is why we believe MTT is a better choice to measure cell survival and proliferation in agar assays.

3)Line 468/469, how long does it take for STASIS to occur?
STASIS generally occurs towards the second week after cell culture is established from breast samples. During STASIS cells display senescent-like features and they do not divide. This period is considered to be overcome when cells start to divide again, as evidenced by an increased slope on growth curves. In figure 3 we have represented the accumulation of population doublings with time of cells derived in our lab from two different donors. For both donors, STASIS started at week 2, and despite the exact duration of this stage depended on the donor, by the 4th week of culture cells started to divide again.

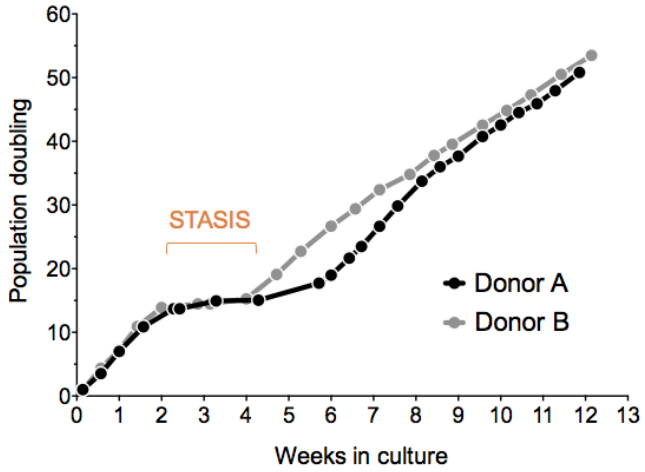


Figure 3. Representative growth curves of cells from two donors. STASIS period is indicated.

In order to clarify this point in the manuscript, a better explanation of STASIS has been now added to the REPRESENTATIVE RESULTS section: "After overcoming STASIS (stress or aberrant signaling induced-senescence, a phenomenon typically observed in mammary epithelial cells *in vitro* that is overcome around 4 weeks after cell culture establishment), cells were…"

4) Line 506, can you confirm the transformation via growth independence of contact with basal lamina via any staining?
The most widely used test to assess the anchor-independent cell growth is the soft-agar assay. If individualized cells in agar can still divide and form colonies some weeks after being seeded, they will become positive colonies after MTT assay. As far as we know, there is no specific staining that identifies cells that can grow with independence of contact with basal lamina and distinguishing them from those that cannot. Cells that can survive in an anchor-independent manner alter the transcription pattern when they are forced to live without anchorage (Sekiguchi and Yamada 2018.

DOI:10.1016/bs.ctdb.2018.02.005). Thus, it is probably necessary to force them to live under these circumstances to manifest their ability.

5)may be provide a table for optimal seed counting and amount matrigel based on your experience?

We thank the reviewer for this suggestion as choosing the optimal plating conditions is a critical point. In the protocol section, we provide the optimal conditions for the formation of 3D structures of mammary cells in 24 well plates (growth area of 1.9 cm^2): 100 µl of Matrigel + 500 µl containing 100.000 cells in medium with 4 % Matrigel per well.

The volume of Matrigel required depends on the size of the well, although an important consideration has to be taken into account: the formation of a meniscus. That is why we highly recommend researchers to use a 24 well plate (or the equivalent well size in the 4 well plate format). As shown in figure 4, wells of lower dimensions (e.g. 96 well plates) require less volume of matrix but the hydrophobic walls cause the Matrigel to form a meniscus. This results on an uneven surface that can alter 3D formation and makes it difficult to examine cells or acini under a microscope. Wells of bigger dimensions than 24 well plates are not commonly used due to the high volume of matrix required, which is expensive (more than 300 € for a 10 ml bottle).

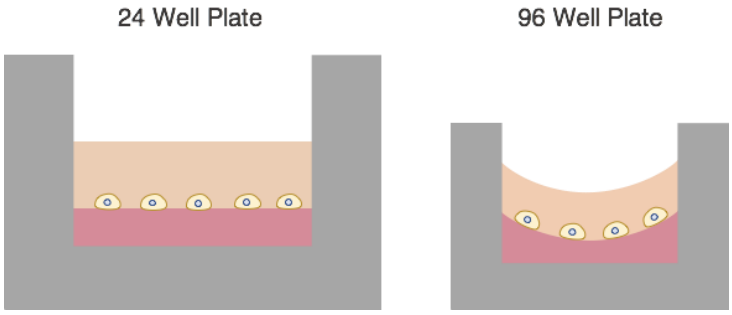


Figure 4. Schematic representation of the basement membrane matrix behavior in wells of different dimensions.

Radiation-Induced Malignant Transformation of Preneoplastic and Normal Breast Primary Epithelial Cells

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