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Quantification of self-renewal in murine mammosphere cultures

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TITLE:

Quantification of Self-Renewal in Murine Mammosphere Cultures

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KEYWORDS:

Myc, mammosphere, growth curve, self-renewal assay, mammary gland, stem cells, progenitors, breast cancer

SUMMARY:

Here, we describe the implementation and interpretation of the results of an in vitro mammosphere self-renewal quantitative assay.

ABSTRACT:

The mammary gland is characterized by extensive regeneration capacity, as it goes through massive hormonal changes throughout the life cycle of a female. The role of mammary stem cells (MaSCs) is widely studied both in the physiological/developmental context and with regards to breast carcinogenesis. In this aspect, ex vivo studies focused on MaSC properties are highly sought after. Mammosphere cultures represent a surrogate of organ formation and have become a valuable tool for both basic and translational research. Here, we present a detailed protocol for the generation of murine primary mammosphere cultures and the quantitation of MaSC growth properties. The protocol includes mammary gland collection and digestion, isolation of primary mammary epithelial cells (MECs), establishment of primary mammosphere cultures, serial passaging, quantitation of mammosphere growth parameters and interpretation of the results. As an example, we present the effect of low-level constitutive Myc expression on normal MECs leading to increased self-renewal and proliferation.

INTRODUCTION:

Isolation and in vitro culture of mammary epithelial stem and progenitor cells have become essential for understanding their properties in mammary cell biology. Elegant lineage tracing and

serial transplantation assays have enabled the study of stem cells (SCs) and other tissue subsets in the context of their in vivo niche. However, this approach is time consuming and requires the generation of reporter mouse models[1-5]. Therefore, in vitro culture and propagation of mammary stem cells (MaSCs) while sparing key stemness features, namely self-renewal and differentiation ability, is one of the biggest challenges in the field. In the last years, the mammosphere assay has been widely used to model both normal mammary tissue and breast cancer growth, to quantify normal or cancer SCs (CSCs) and assess their self-renewal ability as a surrogate reporter of their activity in their respective in vivo context[6-11].

The mammosphere assay is an efficient and cost-effective approach, in which freshly isolated mammary epithelial cells (MECs) are cultured in non-adherent conditions, with the premise that only MaSCs will survive and form spheres in suspension while all the other cell types will die by anoikis. Moreover, the ability to form several generations of mammospheres in serial non-adherent passages is related to the self-renewal ability of the MaSCs[6,9,11]. Here, we describe a detailed protocol of a quantitative mammosphere assay, which was initially developed by Dontu and colleagues[7] as a modification of the pioneering neurosphere assay[12], enabling the growth of putative SCs in non-adherent, serum-free conditions with the addition of appropriate growth factors[7,12].

PROTOCOL:

In vivo procedures were performed in accordance with EU directive 2010/63 and after approval from our institutional ethics committee (Organism for Animal Wellbeing—OPBA) and the Italian Ministry of Health (IACUC Numbers 762/2015 and 537/2017).

1. Murine mammary gland collection and digestion

1.1. For a typical experiment, sacrifice 8-10 weeks-old virgin female mice by CO_2 inhalation. Depending on the aims of the experiment, use 5-30 mice. Place the mice on dissection boards under a hood. Use needles to stretch the forelimbs and wash down the fur of the animal with ethanol.

1.2. Lift the skin with forceps and perform a vertical incision starting at the level of the pelvic area and moving all the way to the cervix, leaving the peritoneum intact. To avoid rupturing the skin or the peritoneum, use round-edged scissors.

1.3. Carefully detach the skin from the body with gentle movements of the scissors across the lateral axis of the body.

1.4. Once the skin is fully detached from the thoracic and abdominal area, perform four incisions across the four limbs of the animal and pin down the skin with needles. Use the scissors and forceps to fully detach the body of the animal from the extended skin.

1.5. Use the forceps to gently lift the mammary fat pads that are now fully exposed and carefully detach them from the skin with the aid of the scissors. Collect the lower thoracic and

abdominal mammary glands of each mouse and immerse them in Dulbecco's phosphate buffered saline (DPBS), in a 50 mL conical tube. Collect up to 20 glands per tube. Keep the tissues on ice.

NOTE: If needed, the glands can remain on ice overnight. This is a safe stopping point. The following steps should be performed under sterile conditions.

1.6. Prepare and filter the digestion medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 200 U/mL collagenase and 100 µg/mL hyaluronidase (see **Table of Materials**).

1.7. Transfer up to 20 glands to a 100 mm Petri dish and mince the tissue using a scalpel or curved scissors. Avoid transferring large volumes of DPBS to the dish.

1.8. Add 10 mL of digestion medium to each Petri dish and transfer the minced tissue to a 50 mL conical tube, using a 25 mL serological pipette.

1.9. Seal the tubes with parafilm and place them on a rotator. Set the rotator at a low speed (0.03 x *g*) and incubate for 2.5 h at 37 °C in a humid atmosphere containing 5% CO_2.

1.10. Visually inspect the suspension before proceeding to the next steps. If large pieces of tissue remain undigested, prolong the incubation at 37 °C for another 30 min.

NOTE: As an alternative, the digestion can be performed overnight at 37 °C, 5% CO_2, using a gentle collagenase/hyaluronidase enzyme mix[13].

2. Isolation of primary murine MECs

2.1. Stop the rotator and remove the tubes from the incubator. Adjust a P1000 tip at the opening of a 5 mL serological pipette. If the suspension passes through the P1000 tip, proceed to the next steps. Otherwise, prolong the incubation at 37 °C for another 30 min.

2.2. Centrifuge at 100 x *g*, for 5 min at 4 °C. Carefully decant the supernatant and resuspend the pellet of each tube in 3 mL of DPBS.

NOTE: The low centrifugation speed allows the removal of lymphocytes and adipose tissue cells. Gentle manipulation is required to avoid dislodging the pellet at this step.

2.3. Filter the cell suspension in each tube separately, using 100, 70 and 40 µm cell strainers. At each filtering step, wash the strainers with 2 mL of DPBS before collecting the pass-through.

NOTE: From this point on, the cell suspensions can be pooled and processed together.

2.4. Centrifuge at 300 x *g*, for 5 min at 4 °C. Carefully decant the supernatant and resuspend the cells in the remaining volume of DPBS.

2.5. Proceed to the red blood cell (RBC) lysis by adding an equal volume of ammonium-chloride-potassium (ACK) lysis buffer (see **Table of Materials**). Mix by pipetting and incubate on ice for up to 5 min.

2.6. Add 10 mL of DPBS and centrifuge at 300 x *g*, for 5 min at 4 °C. Carefully decant the supernatant and visually inspect the pellet. If the pellet is white, proceed to the next step. Otherwise repeat the RBC lysis step (step 2.5).

2.7. Resuspend the cell pellet in 1-5 mL of mammosphere media: mammary epithelial cell growth basal medium (MEBM), supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 0.5 µg/mL hydrocortisone, 2% B27, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF) and 0.4 IU/mL heparin (see **Table of Materials**).

3. Serial mammosphere re-plating

3.1. For the establishment of mammosphere cultures, plate the cells onto non-tissue culture treated (ultra-low adhesion) 6-well plates at a density of 200,000 viable cells/mL in mammosphere medium. Incubate the cells for 7-10 days at 37 °C, 5% CO_2.

3.2. Prepare and filter 1% poly-HEMA solution in 95% ethanol (see **Table of Materials**). Coat ultra-low adhesion 6-well plates for the following passages, by adding 400 µL of 1% poly-HEMA solution per well and allowing the ethanol to dry completely. For better results, repeat the coating twice.

NOTE: The use of non-coated plates during the first passage allows the selective removal of fibroblasts from the culture.

3.3. At the end of the 7-10 days culture, collect the primary mammospheres from all the wells and centrifuge at 300 x *g*, for 5 min at 4 °C.

3.4. Carefully decant the supernatant and proceed to the mechanical dissociation of the mammospheres using a P200 pipette with filtered tips. Pipette for approximately 100 times and visually inspect the suspension for the presence of large spheroids.

NOTE: A short incubation (2-5 min) of the sphere pellet in a low volume (0.2-0.5 mL) of trypsin or accutase at 37 °C, 5% CO_2, can be used to facilitate mechanical dissociation. Prepare fresh mammosphere medium (step 2.7) for the plating of each passage.

3.5. Resuspend in 1-5 mL of fresh mammosphere medium and count the viable cells. If applicable, split the cells in treatment groups or culture conditions.

3.6. Plate 20,000 viable cells/mL on poly-HEMA-coated ultra-low adhesion 6-well plates and

incubate at 37 °C, 5% CO_2.

NOTE: Mammospheres reach their maximum size within 5-6 days after cell plating. When possible, distribute the cells of each sample or condition to multiple wells to obtain technical replicates. Plating density should be kept low to avoid cell aggregation. A maximum of 20,000 cells/mL is recommended for 6-well plates and 5,000 cells/mL for 24-well plates.

3.7. After 5-7 days, count the number of spheres per well. This can be done either manually, using a microscope equipped with a 10X magnification lens, or using digital image analysis (see step 4).

3.8. Collect the mammospheres of each well separately and centrifuge at 300 x *g*, for 5 min at 4 °C.

3.9. Carefully decant the supernatant and proceed to the mechanical dissociation of the mammospheres using a P200 pipette with filtered tips. Pipette for approximately 100 times and visually inspect the suspension for the presence of large spheroids.

3.10. Resuspend in 1 mL of fresh mammosphere medium and count the viable cells. Pool the cells of each sample or condition and repeat steps 3.6-3.10. Record the number of cells plated and the number of spheres and cells counted per well for each passage. Proceed to step 5 for the cumulative growth calculation.

4. Sphere enumeration using digital image analysis (DIA)

4.1. At the end of each passage, scan the entire surface of all the wells and acquire images of the spheres using a digital camera mounted on a stereoscope. Save the images as .tif files.

4.2. Import the stereoscope images as an **Image Sequence** using ImageJ[14]. Set the scale and the type of image to 8-bit.

4.3. Duplicate the stack of images and select **Subtract Background** from the tab **Process** on the menu bar. Check the options **Light background** and **Sliding para**boloid and click on the button **OK**. Process all the images of the stack.

4.4. Select **Adjust** and then **Threshold** from the tab **Image** of the menu bar. By clicking on **Apply**, a dialogue window titled **Convert Stack to Binary** will appear. Select **Default** as method and **Light** as background. Check the box **Calculate threshold for each image** and click on the button **OK**.

4.5. Select sequentially the commands **Watershed**, **Open** and **Erode** from the **Binary** list under the tab **Process** of the menu bar. Process all images of the stack by clicking on the button **Yes** of the dialogue box that appears.

NOTE: These processes allow for visual segmentation of objects that touch, object smoothing and removal of pixels from the edge of the objects.

4.6. Select the function **Analyze Particles** from the menu **Analyze**. Set the minimum size threshold at 10,000 μm^2 and circularity between 0.50 and 1.00. Select the option **Ellipses** from the **Show** drop-down menu. Check **Summarize**, **Exclude on edges** and **In situ Show** and click on the button **OK.** Process all images of the stack.

4.7. Visually inspect the correspondence of the ellipses with spheres and, if needed, correct the particle count accordingly. Sum the counts from all the frames of each well to obtain the total count of mammospheres per well.

5. Cumulative growth curve calculation

NOTE: The number of mammospheres counted in each well at the end of each passage (P_N) reflects the number of mammosphere-initiating cells seeded at the beginning of P_N.

5.1. For each passage (P_N), register the number of plated cells and the number of cells and spheres counted per well. Calculate the sphere size at the end of each passage:
sphere size P_N (cells / sphere) = cells counted P_N / spheres counted P_N

NOTE: The sphere size at P_N is a measure of the proliferative potential of each mammosphere-initiating cell seeded at P_N.

5.2. Infer the number of plated spheres by dividing the number of cells plated for P_N by the sphere size calculated at the end of the previous passage (P_{N-1}):
spheres plated P_N = cells plated P_N / sphere size P_{N-1}

NOTE: By convention, the sphere size is assumed stable during the first passage of the culture (i.e., sphere size P_0 = sphere size P_1). If multiple wells are used as technical replicates, use the average sphere size at P_{N-1} as denominator.

5.3. Calculate the cumulative cell and sphere number for each well per passage:
cumulative number P_N = (count P_N / plated P_N) X cumulative number P_{N-1}.

NOTE: By convention, cumulative number P_0 = plated P_1. If multiple wells are used as technical replicates, calculate the average cumulative number per sample or condition for each passage.

5.4. Plot the data points on a semi-logarithmic scale. Display the passage number (P_0 to P_N) on the x axis (linear scale) and the cumulative cell or sphere number on the y axis (logarithmic scale).

5.5. Fit an exponential trend-line to the data points and calculate the coefficient of determination (R^2) to measure the goodness of the fit.

NOTE: The trend-line fitted to the data points should approximate an exponential curve, as expected for a cell population that grows or dies with a constant rate. R^2 takes values between 0 and 1, with values closest to 1 indicating a better fit.

5.6. Depict the equation of the trend-line as a natural exponential function to infer the growth rate (GR) of the culture:

$y = y_0 e^{(GR)x}$, where y_0 is the value of y when $x = 0$.

REPRESENTATIVE RESULTS:

Myc overexpression in normal MECs, leads to an increased frequency of mammosphere initiating cells. This is achieved through a double mechanism: Myc increases the rate of MaSC symmetric divisions and the frequency of progenitor reprogramming into new MaSCs[11]. To test the effect of low constitutive Myc expression, we used the Rosa26-MycER transgenic mouse model, in which Myc activity can be induced by 4-hydroxytamoxifen (4-OHT)[15]. We first plated the MECs on ultra-low adhesion 6-well plates to remove fibroblasts, in the absence of 4-OHT. After the first passage, we split the culture in two: two wells were kept untreated (control) and two wells were treated with 200 nM 4-OHT (MycER). We counted the sphere and cell numbers of 5 consecutive passages for three independent experiments (**Table 1**). The cumulative cell and sphere numbers per passage are shown on **Table 2**. Induction with 4-OHT leads to increased sphere and cell growth rates, as shown in **Figure 1**.

FIGURE AND TABLE LEGENDS:

Figure 1: **Representative results**. Cumulative sphere (**A**) and cell (**B**) growth curves of control and MycER mammospheres. Mean and standard deviation of 3 independent experiments are shown.

Table 1: Numbers of spheres counted and numbers of cells plated and counted at each passage.

Table 2: Calculation of plated sphere numbers and cumulative sphere and cell numbers.

DISCUSSION:

Here, we describe a protocol for the quantitative description of MaSC growth properties in vitro. As an example, we present the effect of low-level constitutive Myc expression on normal murine MaSCs. This approach, however, can be equally applied to various contexts. Human or murine primary cells, as well as established cell lines, can be cultured in anchorage independent conditions to establish mammosphere cultures that can be serially passaged. Gene overexpression and RNA interference can be easily introduced in the protocol with the addition of a viral transduction step at the end of the first passage (after step 3.5). Alternatively, cells can be infected in adhesion and then plated as mammospheres.

A critical aspect of the assay presented here is the seeding cell density, which should be low enough to avoid the generation of aggregates interfering with the interpretation of the results[16,17]. The morphology of the mammospheres can be informative to resolve this ambiguity. Only compact, round spheres should be enumerated at the end of each passage. Both the circularity of the spheroids and the size should be taken into consideration. Using the automated

process of the DIA, this step is ensured with the appropriate thresholds in an objective and absolute manner. Often, progenitors will form acinar structures or smaller clusters of cells which should be excluded from the mammosphere counts. As a rule of thumb, we use a threshold of 100 µm diameter. Finally, care should be taken to avoid the transfer of intact or non-fully dissociated mammopsheres from one passage to the next. On the other hand, excess pipetting will lead to increased cell death. Thus, if such difficulties are encountered, we recommend using mild trypsinization or accutase treatment and passing the dissociated spheres through a 40 µm strainer to ensure the generation of single-cell suspensions.

Sphere forming efficiency (SFE) has been used alternatively, as a surrogate for SC or CSC quantitation ex vivo in mammosphere cultures. SFE is indeed a measure of stem-like cells in a given cell population. However, it represents a less conscientious approach since it provides information only at distinct time points. The calculation of cumulative sphere numbers and the generation of cumulative growth curves, instead, enables the inference of the growth rate of the culture from the initial cell seeding step until the culture exhausts or, in the case of immortalized cultures, for the desired number of passages. The assessment of growth properties allows the evaluation of the deviation from the exponential growth through the coefficient R^2 and, at a second step, the assessment of the GR value itself.

Importantly, cumulative mammosphere growth curves can be used to evaluate the effect of small molecule inhibitors or other chemotherapeutic drugs selectively at the CSC level[6,11]. Contrary to normal primary mammospheres, which functionally exhaust in 5-7 passages, tumor mammospheres tend to expand indefinitely. This feature is linked to the unlimited CSC self-renewal ability. The effects on proliferation and CSC self-renewal can be uncoupled through the generation of tumor cell and mammosphere growth curves, respectively. A CSC-specific effect is expected to result in a decrease in the cumulative mammosphere growth rate, with or without effect on the cumulative cell growth rate[6,11].

Finally, another area of interest is the one of adult tissue SC reprogramming. Fully grown mammospheres consist of a phenotypically heterogeneous cell population, in which only a minor fraction retains stem-like features, including mammosphere-initiating ability and mammary gland regeneration upon transplantation in vivo[6,9,11,18,19]. Mammary progenitors can be thus isolated either using in vitro label-retaining assays[6,9,11] or, ex vivo, using established surface markers[2,3]. Notably, mammary progenitors do not survive anoikis and are unable to form mammospheres. Enforced Myc expression has been shown to confer mammosphere initiation potential to mammary progenitors isolated as PKHneg[11], resulting in the generation of a culture that can be passaged indefinitely. Similarly, interference of negative regulators of physiological reprogramming can be tested using the same assay. In this context, a common issue that may arise is the limited number of cell input. If the cell input is lower than 10,000 cells, we recommend seeding in 24-well plates (maximum 5,000 viable cells/mL). Nevertheless, anchorage independent culture conditions can be proven to be too harsh for scoring reprogramming, especially in cases where the reprogramming effect is not immediate. In such cases, the use of a supportive matrix and 3-dimensional organoid cultures could be more appropriate[20].

Overall, the mammosphere assay is a cost-effective option that can be easily employed for scoring stem-like properties in normal and tumoral MEC populations. The quantitative approach taken in this protocol facilitates the comparisons between cultures carried out in different conditions or exposed to diverse stimuli. When followed rigorously, it provides a relatively simple ex vivo model system that permits uncoupling of the multiple players that define stem properties in vivo, offering the possibility of more detailed mechanistic studies.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Kordon, E. C. & Smith, G. H. An entire functional mammary gland may comprise the progeny from a single cell. *Development.* **125** (10), 1921-1930, (1998).

2 Shackleton, M. et al. Generation of a functional mammary gland from a single stem cell. *Nature.* **439** (7072), 84-88, (2006).

3 Stingl, J. et al. Purification and unique properties of mammary epithelial stem cells. *Nature.* **439** (7079), 993-997, (2006).

4 van Amerongen, R., Bowman, A. N. & Nusse, R. Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. *Cell Stem Cell.* **11** (3), 387-400, (2012).

5 Van Keymeulen, A. et al. Distinct stem cells contribute to mammary gland development and maintenance. *Nature.* **479** (7372), 189-193, (2011).

6 Cicalese, A. et al. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell.* **138** (6), 1083-1095, (2009).

7 Dontu, G. et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & Development.* **17** (10), 1253-1270, (2003).

8 Grimshaw, M. J. et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. *Breast Cancer Research.* **10** (3), R52, (2008).

9 Pece, S. et al. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. *Cell.* **140** (1), 62-73, (2010).

10 Ponti, D. et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Research.* **65** (13), 5506-5511, (2005).

11 Santoro, A. et al. p53 Loss in Breast Cancer Leads to Myc Activation, Increased Cell Plasticity, and Expression of a Mitotic Signature with Prognostic Value. *Cell Reports.* **26** (3), 624-638 e628, (2019).

12 Reynolds, B. A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science.* **255** (5052), 1707-1710, (1992).

13 Gao, H. et al. Murine mammary stem/progenitor cell isolation: Different method matters? *Springerplus.* **5** 140, (2016).

14 Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods.* **9** (7), 671-675, (2012).
15 Murphy, D. J. et al. Distinct thresholds govern Myc's biological output in vivo. *Cancer Cell.* **14** (6), 447-457, (2008).
16 Bailey, P. C. et al. Single-Cell Tracking of Breast Cancer Cells Enables Prediction of Sphere Formation from Early Cell Divisions. *iScience.* **8** 29-39, (2018).
17 Lombardo, Y., de Giorgio, A., Coombes, C. R., Stebbing, J. & Castellano, L. Mammosphere formation assay from human breast cancer tissues and cell lines. *Journal of Visualized Experiments.* 10.3791/52671 (97), (2015).
18 Peng, T., Qinghua, M., Zhenning, T., Kaifa, W. & Jun, J. Long-term sphere culture cannot maintain a high ratio of cancer stem cells: a mathematical model and experiment. *PLoS One.* **6** (11), e25518, (2011).
19 Smart, C. E. et al. In vitro analysis of breast cancer cell line tumourspheres and primary human breast epithelia mammospheres demonstrates inter- and intrasphere heterogeneity. *PLoS One.* **8** (6), e64388, (2013).
20 Panciera, T. et al. De Novo Generation of Somatic Stem Cells by YAP/TAZ. *Journal of Visualized Experiments.* 10.3791/57462 (135), (2018).
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Figure 1

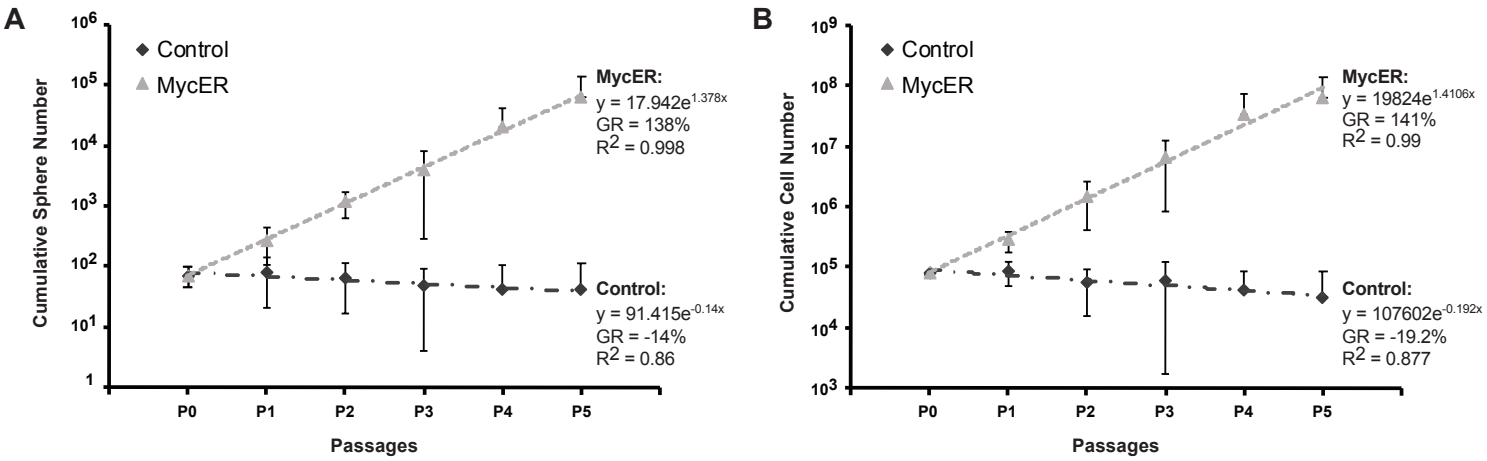

Table 1

		1st plating			2nd plating			3rd plating			4th plating			5th plating		
		Cells Plated	**Cell Count**	**Sphere Count**	**Cells Plated**	**Cell Count**	**Sphere Count**	**Cells Plated**	**Cell Count**	**Sphere Count**	**Cells Plated**	**Cell Count**	**Sphere Count**	**Cells Plated**	**Cell Count**	**Sphere Count**
Exp1	**Control**	77000	50000	31	65000	58500	41	57000	30000	32	30000	22000	5	22000	0	0
		77000	81000	41	65000	55000	23									
	MycER	77000	310000	193	80000	375000	323	80000	380000	217	80000	220000	223	80000	170000	155
		77000	110000	142	80000	505000	396	80000	270000	149	80000	290000	194	80000	250000	160
Exp2	**Control**	75000	75000	71	60000	17000	34	28000	45000	29	45000	13000	2	13000	0	0
		75000	47000	45	60000	11000	47									
	MycER	75000	200000	188	80000	225000	277	80000	230000	155	80000	210000	211	80000	100000	95
		75000	250000	192	80000	202500	283	80000	305000	185	80000	160000	237	80000	100000	133
Exp3	**Control**	82500	130000	121	80000	45000	105	80000	110000	86	80000	58500	75	58500	58500	78
		82500	125000	177	80000	71250	42									
	MycER	82500	325000	457	80000	610000	327	80000	367000	309	80000	500000	260	80000	115000	146
		82500	475000	463	80000	455000	392	80000	415000	204	80000	470000	295	80000	185000	161

Table 2

		Exp1						Exp2						Exp3					
		Plated		Counted		Cumulative		Plated		Counted		Cumulative		Plated		Counted		Cumulative	
		Cells	Spheres	Cells	Spheres	Cells	Spheres	Cells	Spheres	Cells	Spheres	Cells	Spheres	Cells	Spheres	Cells	Spheres	Cells	Spheres
P1	**Control**	77000	48	50000	31	50000	31	75000	71	75000	71	75000	71	82500	77	130000	121	130000	121
		77000	39	81000	41	81000	41	75000	72	47000	45	47000	45	82500	117	125000	177	125000	177
	MycER	77000	43	310000	193	310000	193	75000	71	200000	188	200000	188	82500	97	325000	457	325000	457
		77000	43	110000	142	110000	142	75000	71	250000	192	250000	192	82500	97	475000	463	475000	463
P2	**Control**	65000	36	58500	41	58950	41	60000	57	17000	34	17283	35	80000	93	45000	105	71719	167
		65000	36	55000	23	55423	23	60000	57	11000	47	11183	48	80000	93	71250	42	113555	67
	MycER	80000	64	375000	323	984375	848	80000	68	225000	277	632813	779	80000	92	610000	327	3050000	1635
		80000	64	505000	396	1325625	1040	80000	68	202500	283	569531	796	80000	92	455000	392	2275000	1960
P3	**Control**	57000	32	30000	32	30098	32	28000	81	45000	29	22875	15	80000	101	110000	86	127375	100
	MycER	80000	65	380000	217	5486250	3133	80000	105	230000	155	1728369	1165	80000	54	367000	309	12214219	10284
		80000	65	270000	149	3898125	2151	80000	105	305000	185	2291968	1390	80000	54	415000	204	13811719	6789
P4	**Control**	30000	32	22000	5	22072	5	45000	29	13000	2	6608	1	80000	63	58500	75	93143	119
	MycER	80000	45	220000	223	12903516	13079	80000	51	210000	211	5276692	5302	80000	52	500000	260	81331055	42292
		80000	45	290000	194	17009180	11379	80000	51	160000	237	4020337	5955	80000	52	470000	295	76451191	47985
P5	**Control**	22000	5	0	0	0	0	13000	2	0	0	0	0	58500	75	58500	78	93143	124
	MycER	80000	65	170000	155	31782239	28978	80000	97	100000	95	5810643	5520	80000	46	115000	146	113405989	143976
		80000	65	250000	160	46738586	29913	80000	97	100000	133	5810643	7728	80000	46	185000	161	182435722	158768

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
ACK lysis buffer	Lonza	10-548E	Ammonium-chloride-potassium lysis bufer, 100 mL.
B27	Invitrogen	17504-044	B27 supplement 50X (10 mL). Final concentration 2% v/v.
bFGF	Peprotech	100-18B	Human recombinant fibroblast growth factor - basic, 50 µg. Stock solution 100 µg/µL in Tris 5 mM pH 7.6. Final dilution 0.02% v
Collagenase	Sigma	C2674	Collagenase from *Clostridium histolyticum* . Type I-A, lyophilized powder, 1 g. Stock 20,000 U/mL in DMEM. Final dilution 1% v/v
DMEM	Lonza	12-614F	Dulbecco's modified Eagle's medium
DPBS	Microgem	S17859L0615	Dulbecco's phosphate buffered saline
EGF	Tebu-Bio	AF-100-15	Recombinant human epithelial growth factor. Stock solution 100 µg/mL in sterile dH_2O. Final dilution 0.02% v/v.
Glutamine	Lonza	17-605E	L-Glutamine, 200 mM. Final dilution 1% v/v.
Heparin	PharmaTex	34692032	Stock concentration 5,000 IU/mL. Final dilution 0.008% v/v.
Hyaluronidase	Sigma	H4272	Type IV-S, powder, 750-3,000 U/mg solid, 30 mg. Stock solution 10 mg/mL in sterile dH_2O. Final dilution 1% v/v.
Hydrocortisone	Sigma	H0888	Stock concentration 100 µg/mL. Final dilution 0.5% v/v.
Insulin	SAFCBiosciences	91077C	Insulin, human recombinant, dry powder, 250 mg. Stock concentration 1 mg/mL. Final dilution 0.5% v/v.
Low attachment 6-well plates	Corning	351146	Sterile 6-well not treated cell culture plates with clear flat bottom and lid.
MEBM	Lonza	CC-3151	Mammary epithelial cell growth basal medium
Penicllin-Streptomycin mixture	Lonza	17-602F	Contains 10,000 U potassium penicillin and 10,000 µg streptomycin sulfate per mL in 0.85% saline. Final dilution 1% v/v.
Poly-HEMA	Sigma	P3932	Dissolve in 95% EtOH overnight at 55 ^{o}C. Stock concentration 12% w/v. Final dilution 1% v/v in 95% EtOH. Filter (0.22 µm) befor

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Author(s):	Thalia Vlachou, Xieraili Aobuli, Errico D'Elia, Angela Santoro, Maria Cristina Moroni and Pier Giuseppe Pelicci

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Editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank the Editor for the kind suggestion. We have carefully read the manuscript and corrected all spelling and grammar mistakes we identified.

2. Please obtain explicit copyright permission to reuse any figures (including TOC) from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The protocol presented here is routinely used in our lab and was also implemented in our recent publication in *Cell Reports*[1]. However, the growth curves shown on Figure 1 of the present manuscript have been calculated and drawn based on different datasets (provided in Table 1) than the ones used in the Cell Reports research article. Therefore, Figure 1 is original and not adapted from a previous publication, lifting the need to request any copyright permission.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

The numbering format has been adjusted according to JoVE instructions and all bullets and dashes have been removed from the manuscript.

4. Please highlight complete sentences (not parts of sentences) for filming.

We thank the Editor for pointing out this requirement. We have now taken care of highlighting complete sentences for filming.

5. Please do not abbreviate journal titles for references.

We apologize for the use of abbreviated journal titles in the bibliography. We have now introduced full journal names for all references through EndNote.

Reviewers' comments:

Reviewer #1:

This is a very useful contribution and I find no objections to its publication in Jove. In spite of current advancements on organoids from mouse mammary gland (e.g., ref 17) and human mammary tumors, mammosphere cultures remains extremely useful and quantitative tools to measure self renewal and the team was among the pioneers of this methodology.

We thank Reviewer #1 for dedicating the time to carefully read our manuscript and to provide valuable feedback.

Reviewer #2:

1- I invite the authors to increase number of details and options (i.e. motion rpm, water bath or incubation times).

We thank Reviewer #2 for bringing this issue to our attention. We have carefully read the protocol and added all missing information regarding motion rpm and incubation times. We have also added the option of overnight digestion with gentle collagenase/hyaluronidase enzyme mixes to provide more flexibility to the readers. We hope to have addressed all points of interest.

2- the cell density should be reduced.

We agree with Reviewer #2 that keeping a low cell density is critical for the serial re-plating mammosphere assay (we kindly refer to Discussion, paragraph 2). In fact, we consider 20,000 cells/mL as the maximum cell density that can be employed for such experiments, in order to accommodate additional downstream applications (e.g. expression analyses). To ensure greater clarity, we have now included an additional note after step 3.6 in the protocol.

3- Mechanical dissociation could be avoided and replaced with ACCUTASE treatment, or at least discuss these options.

We thank Reviewer #2 for highlighting this point. We have now included a note after step 3.4 to provide the missing information and also mention the alternative options in our Discussion, paragraph 2.

Moreover, these points from literature should be discussed/updated:

Peng et al. in 2011.[2]… found that the CSC ratio in long-term sphere culture presented as gradually decreased drift and might be stable at a lower level.

Moreover, the group of Lopez 2013[3] in their article state:

Some studies have shown an inability to serially cultivate normal mammospheres beyond five passages[4], whilst others have demonstrated an inability to maintain a high ratio of CSCs (CD44+/CD24−) in long-term sphere culture, suggesting limited self-renewal capacity[2]. Spheres are known to express markers of differentiation[5], further suggesting they may not universally 'enrich' for stem cells. Other studies are emerging that convincingly demonstrate sphere formation actually reverses CSC phenotype in some cell lines[6].

We thank Reviewer #2 for the suggestion. We have now better clarified this point, by acknowledging the existence of inter- and intra-mammosphere phenotypic heterogeneity

and that only a minor fraction of the culture retains the ability to form new spheres *in vitro* and/or form a functional organ *in vivo*. Please refer to our Discussion section, paragraph 5.

References:

1 Santoro, A. *et al.* p53 Loss in Breast Cancer Leads to Myc Activation, Increased Cell Plasticity, and Expression of a Mitotic Signature with Prognostic Value. *Cell Reports.* **26** (3), 624-638 e628, (2019).

2 Peng, T., Qinghua, M., Zhenning, T., Kaifa, W. & Jun, J. Long-term sphere culture cannot maintain a high ratio of cancer stem cells: a mathematical model and experiment. *PLoS One.* **6** (11), e25518, (2011).

3 Smart, C. E. *et al.* In vitro analysis of breast cancer cell line tumourspheres and primary human breast epithelia mammospheres demonstrates inter- and intrasphere heterogeneity. *PLoS One.* **8** (6), e64388, (2013).

4 Dey, D. *et al.* Phenotypic and functional characterization of human mammary stem/progenitor cells in long term culture. *PLoS One.* **4** (4), e5329, (2009).

5 Dontu, G. *et al.* In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & Development.* **17** (10), 1253-1270, (2003).

6 Matilainen, H., Yu, X. W., Tang, C. W., Berridge, M. V. & McConnell, M. J. Sphere formation reverses the metastatic and cancer stem cell phenotype of the murine mammary tumour 4T1, independently of the putative cancer stem cell marker Sca-1. *Cancer Letters.* **323** (1), 20-28, (2012).