Video Article

Spheroid Assay to Measure TGF-β-induced Invasion

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Abstract

TGF-β has opposing roles in breast cancer progression by acting as a tumor suppressor in the initial phase, but stimulating invasion and metastasis at later stage[1,2]. Moreover, TGF-β is frequently overexpressed in breast cancer and its expression correlates with poor prognosis and metastasis [3,4]. The mechanisms by which TGF-β induces invasion are not well understood.

TGF-β elicits its cellular responses via TGF-β type II (TβRII) and type I (TβRI) receptors. Upon TGF-β-induced heteromeric complex formation, TβRII phosphorylates the TβRI. The activated TβRI initiates its intracellular canonical signaling pathway by phosphorylating receptor Smads (R-Smads), i.e. Smad2 and Smad3. These activated R-Smads form heteromeric complexes with Smad4, which accumulate in the nucleus and regulate the transcription of target genes[5]. In addition to the previously described Smad pathway, receptor activation results in activation of several other non-Smad signaling pathways, for example Mitogen Activated Protein Kinase (MAPK) pathways[6].

To study the role of TGF-β in different stages of breast cancer, we made use of the MCF10A cell system. This system consists of spontaneously immortalized MCF10A1 (M1) breast epithelial cells[7], the H-RAS transformed M1-derivative MCF10AneoT (M2), which produces premalignant lesions in mice[8], and the M2-derivative MCF10CA1a (M4), which was established from M2 xenografts and forms high grade carcinomas with the ability to metastasize to the lung[9]. This MCF10A series offers the possibility to study the responses of cells with different grades of malignancy that are not biased by a different genetic background.

For the analysis of TGF-β-induced invasion, we generated homotypic MCF10A spheroid cell cultures embedded in a 3D collagen matrix *in vitro* (Fig 1). Such models closely resemble human tumors *in vivo* by establishing a gradient of oxygen and nutrients, resulting in active and invasive cells on the outside and quiescent or even necrotic cells in the inside of the spheroid[10]. Spheroid based assays have also been shown to better recapitulate drug resistance than monolayer cultures[11]. This MCF10 3D model system allowed us to investigate the impact of TGF-β signaling on the invasive properties of breast cells in different stages of malignancy.

Video Link

The video component of this article can be found at https://www.jove.com/video/3337/

Protocol

1. Preparation of the methocel solution (500 mL)

1. Autoclave 6 g of methylcellulose in a 500 ml bottle containing a magnetic stirrer.
2. Dissolve the methylcellulose in 250 ml of preheated DMEM/F12 without serum (60°C) for 20 min.
3. Add 250 ml of DMEM/F12 (RT) containing 10% horse serum. Mix overnight (4°C).
4. Clear the solution by centrifugation (5000g, 2h, 4°C).
5. Take the clear highly viscous solution to a new tube (about 90-95% of the stock solution).
6. Store at 4°C until use.

2. Spheroid culture

1. Prepare a 20% methocel solution (10 ml methocel and 40 ml growth medium).
2. Wash cells twice with PBS, add 0.1% trypsin and incubate cells at 37 °C
3. Resuspend cells in growth medium and count the cells.
4. Prepare a suspension of 10 000 cells per ml in 20% methocel.
5. Add the cell suspension to the 96 well round bottom suspension plates (100 µl per well).
6. Next day, check the spheroid formation in the suspension plates. Spheroids will be ready for use after 1-3 days.

3. Preparation of collagen

1. Prepare on ice a solution of 8 ml collagen, 1 ml 10x PBS, 1 ml 0.1 N NaOH and 3 drops 0.1 N HCl. Check if pH is 7.4 by spotting some of the solution on pH indicator strips. Adjust pH if out of range.
2. Add 50 µl of neutralized collagen solution in the wells of a 96 well plate
3. Incubate at 37°C until the collagen is solidified (90 min).
4. Prepare methocel-collagen-ligand solutions on ice: for each ligand pipette 1 part of of collagen solution. Add 20 ng per ml of collagen TGF-β. After dilution with methocel and diffusion over the different layers, the final concentration of TGF-β will be 5 ng/ml Mix by vortexing. Add 1 part of methocel- solution. Mix by vortexing.

4. Spheroid embedding

1. Place a 200 µl tip which has approximately 5 mm of the tip cut off, on a 200 µl pipette. Suck up one spheroid with the pipette and carefully dispense the medium into an empty plate. Watch if the spheroid stays inside the tip. The spheroid is visible as a white dot. Without releasing the plunger, suck up 100 µl collagen-methocel mixture and dispense the spheroid in the collagen mixture into a collagen-coated 96 well. Do this for 12 spheroids for each condition.
2. Let collagen solidify at least 30 minutes at 37°C
3. Remove air bubbles with a 200 µl tip.
4. Add 50 µl of medium with 1.6 % serum and inhibitors dissolved in DMSO. For SB-431542 add 4 µl of stock solution (10 mM) to 1 ml of medium. After diffusion, the final concentration of the inhibitor will be 10 µM. TGF-β and inhibitors will stay the course of the experiment. Take pictures of the spheroids under the microscope using 40X magnification.
5. After 48h of stimulation with TGF-β and/or inhibitors, take pictures of the spheroids under the microscope using 40X magnification.
6. Measure the area of the invading spheroids after 2 days and subtract the starting area. Measuring the area of a spheroid: Open the picture from the spheroid in Adobe Photoshop Extended (figure 2A) and select the Quick Selection tool (figure 2B). The mouse pointer changes into a circle with a 'plus' (+) sign (figure 2C). While dragging the cursor over the spheroid, Photoshop will recognize the borders of the spheroid. If an area outside the spheroid is selected, this region can be excluded from the selection by pressing the Alt key or by using the negative selection tool which is indicated by a (-) sign in the toolbar. The cursor changes into a circle with a 'minus' (-) sign and by dragging the cursor over the wrongly selected area, this area is removed from the selection (figure 2D). If necessary, multiple regions can be selected by pressing the Shift button. To work more accurately, the size of the mouse pointer can be adjusted by changing the brush diameter in the toolbar (figure 2E). When the whole spheroid is selected, the area of the selection is calculated via ANALYSIS-MEASURE in the menu bar or by pressing Ctrl + Shift + M (figure 2F). The measurement recording window will appear showing the file name and the area of the selection in pixels, as well as other data (figure 2G). If multiple selections are measured, the first line shows the sum of all the areas. The measurements of the individual selections are presented in the lines below. The data of all the measurements can be exported into a text file and opened in Excel.

5. Representative results:

An example of the spheroid assay with MCF10A1 (M1) normal breast epithelial cells, H-RAS transformed MCF10AneoT (M2) cells and M2-derived MCF10CA1a (M4) is given in Figure 3. M1 cells showed only weak invasion without stimulation, but invaded significantly better upon stimulation by TGF-β. In contrast, the RAS-transformed M1-derivative M2 invaded already efficiently without addition of stimuli, and this was further increased 4-5 fold upon TGF-β treatment. M4 cells showed the strongest invasion, both with and without TGF-β addition.

Next, we examined whether we could interfere with TGF-β-induced invasion in this spheroid model using chemical inhibitors. For this purpose we used SB-431542, an ATP analogue and selective inhibitor of the kinase activity of TβRI, as well as activin type IB receptor (ActRIB) and activin receptor-like kinase (ALK)7[12]. As expected, SB-431542 potently inhibited TGF-β-induced invasion of M1, M2 and M4 cells (Fig 4), indicating that TGF-β-induced invasion is TβRI-kinase dependent. In addition, the basal invasion of the spheroids was also strongly inhibited, suggesting that basal invasion is also dependent on TGF-β.

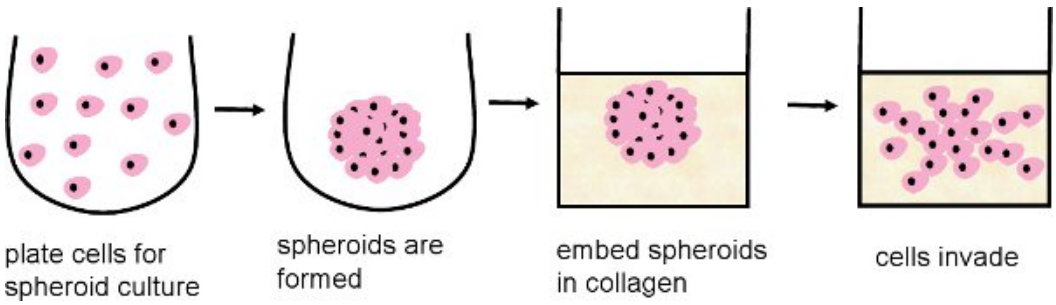


Figure 1. Flow chart of the 3D spheroid assay. First, cells are plated under non-adherent conditions in u-shaped suspensions plates. Cells aggregrate into multcellular spheroids and can be embedded into a collagen matrix. Into this collagen matrix, they are able to invade.

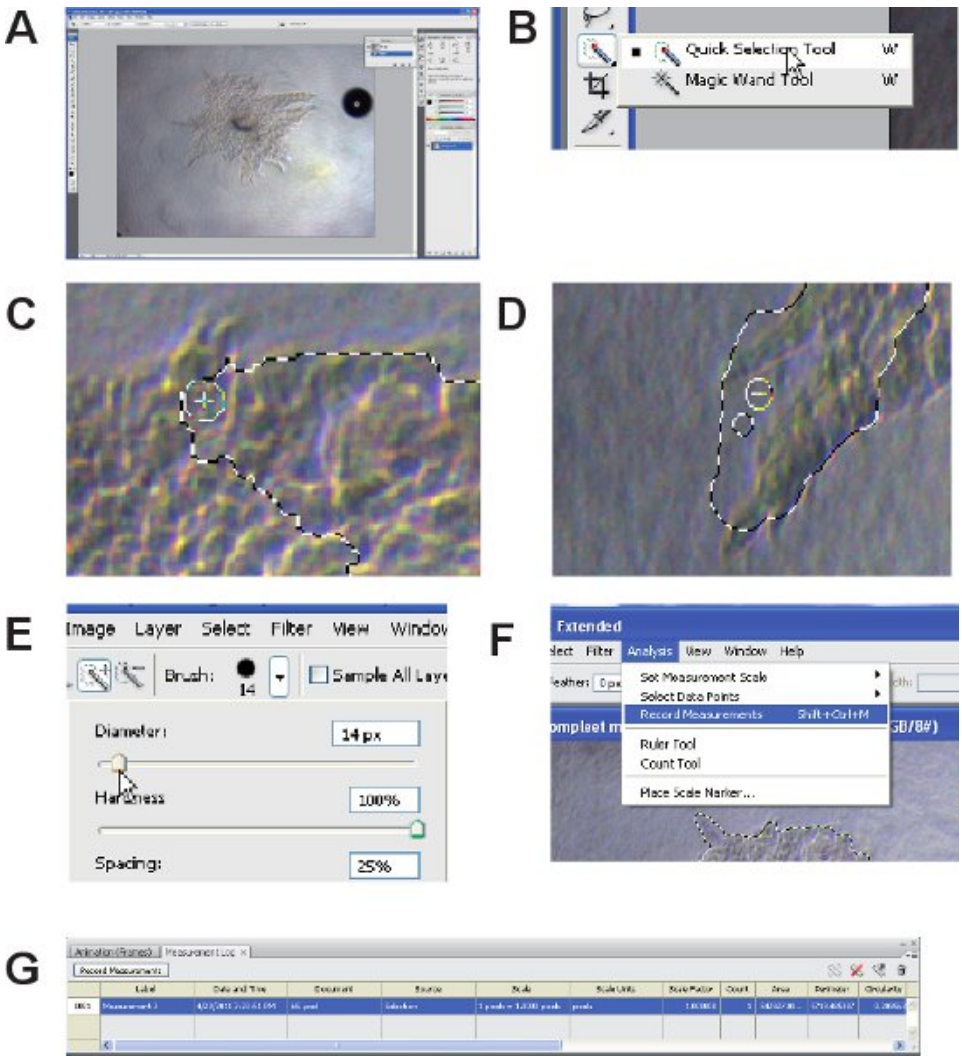


Figure 2. Quantification of the area of the spheroids using Adobe Photoshop Extended. (A) The picture of the spheroid is opened in Adobe Photoshop Extendend (B) Selection of the Quick Selection tool (C) Dragging the cursor over the spheroid to select the area of the spheroid (D) Removal of a wongly included area using the negative Quick Selection tool (E) Adjustment of the brush size of the Quick Selection tool to more accurately select the area (F) Selection of the measurement command to record (G) Example of a measurement record.

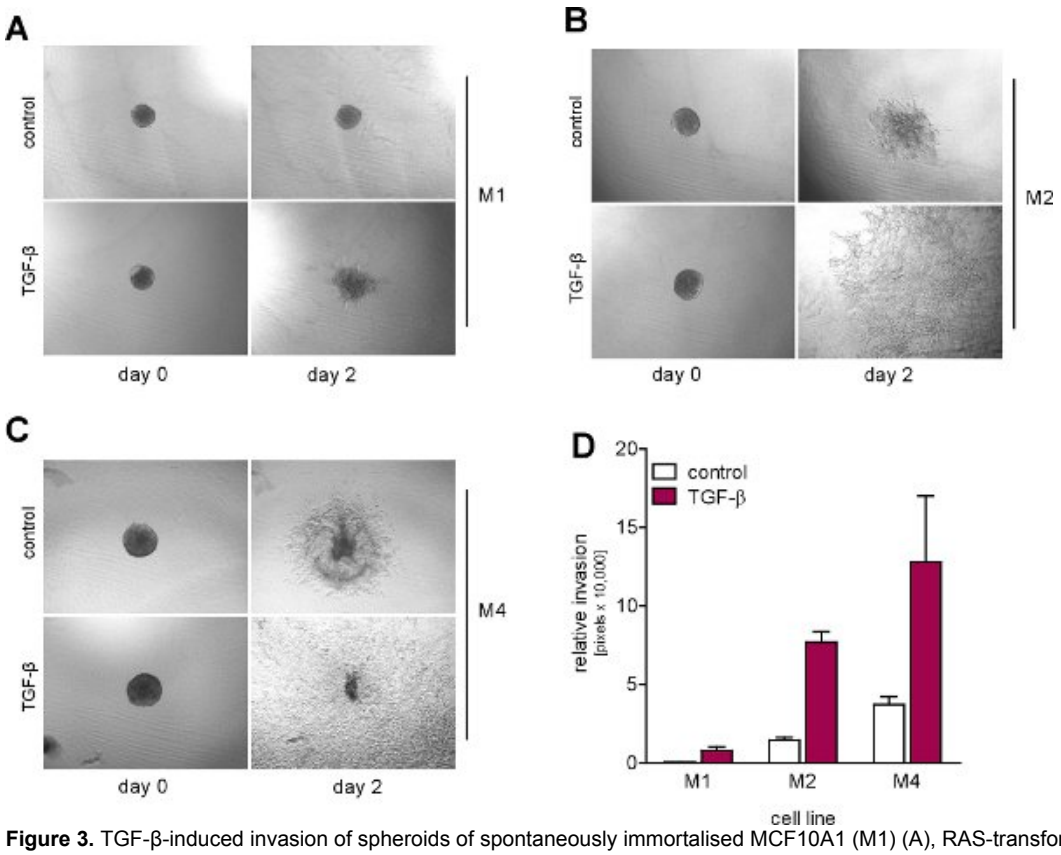
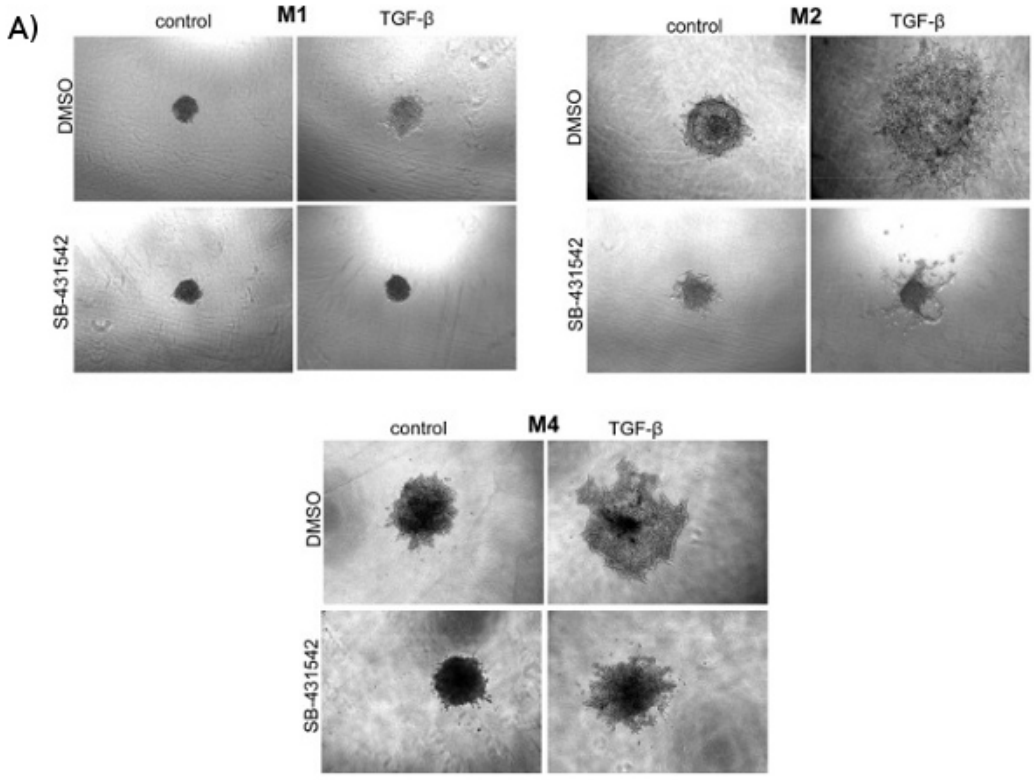


Figure 3. TGF-β-induced invasion of spheroids of spontaneously immortalised MCF10A1 (M1) (A), RAS-transformed MCF10AneoT (M2 (B) and its metastatic derivative MCF10CA1a (M4) (C). M1, M2 and M4 spheroids embedded into collagen were treated with 5ng/ml of TGF-β for 48h as indicated. **A-C** Representative pictures taken at the day of embedding and two days later. **D** Relative invasion was quantified as area difference on day 2 minus day 0. The results are expressed as mean ± s.d. Adapted from[13].



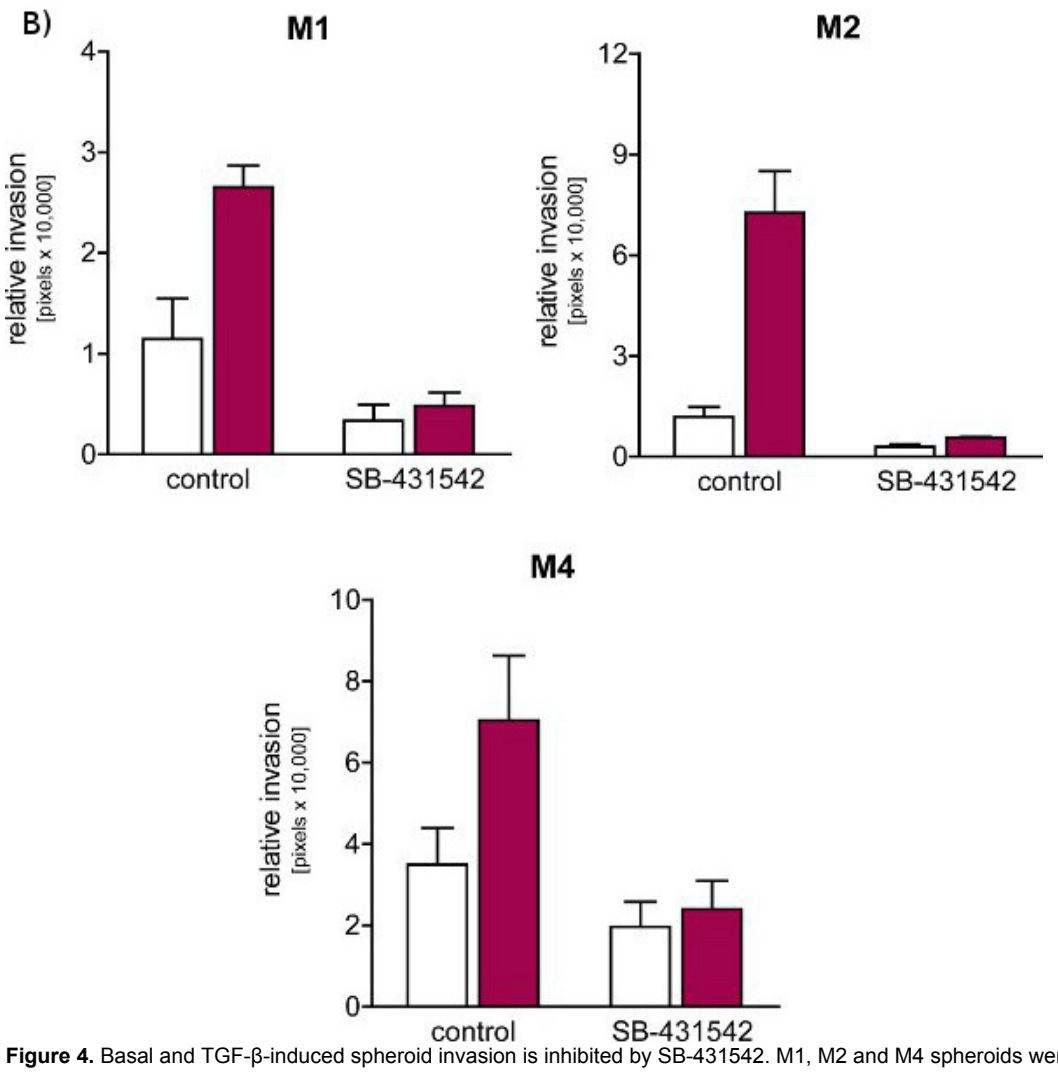


Figure 4. Basal and TGF-β-induced spheroid invasion is inhibited by SB-431542. M1, M2 and M4 spheroids were embedded into collagen and treated with 5ng/ml TGF-β and/or 10 μM SB-431542 as indicated. Representative pictures taken after 2 days (A). Relative invasion was quantified as area difference on day 2 minus day 0 (B). The results are expressed as mean ± s.d. Adapted from[13].

Discussion

We established a spheroid model in which MCF10A1 cells and its malignant derivatives invade into a collagen matrix in a TGF-β-dependent manner. First, spheroids are formed in 96-wells round bottom suspension plates in the presence of methylcellulose. Of course, also U-shaped poly-HEMA coated plates can be used for this purpose. Methylcellulose is absolutely required in this step, since cells will die in the absence of methylcellulose. TGF-β is added directly to the collagen-methocel mixture since we found that cells responded less well to the TGF-β when this factor was added to the medium op top of the collagen. However, it is advisable to add chemical inhibitors dissolved in DMSO not directly into the collagen, since the DMSO precipitates in the icecold collagen-methocel mixture. Therefore, we add these compounds in the medium on top of the collagen.

Subsequently, cells are embedded in a collagen 3-dimensional environment to allow them to invade into the collagen matrix. When comparing the invasive properties of the different MCF10A1 cell lines we found that basal invasion as well as TGF-β-induced invasion increased from the relatively benign M1 cells, to higher levels in the RAS-transformed M2 derivative, to even higher levels in the metastatic M4 variant. Thus, the invasive properties correlated with the relative state of aggressiveness of these three cell lines[7,8,14].

The embedding of the spheroid into the collagen is a critical step in the assay. The detection of the spheroid in the pipette tip might be difficult when performing the assay for the first few times. One can avoid this by collecting the spheroids in a tube, spinning them down, removing supernatant and resuspend the spheroids in collagen-methocel mixture. This requires more spheroids as input as around 50% of the spheroids is lost during this process. Furthermore, multiple spheroids may end up in one well and might lie too close to each other to analyse. In addition, it is preferred to have not more then one spheroid per well to avoid the possibility that spheroids may interfere with each other (e.g. by secreting growth factors). Therefore the resuspension step is a critical step using this method.

A limitation of this assay is that we analyze a 3 dimensional assay in a 2 dimensional plane. As can be seen in Figures 3 and 4, most cells usually invade in the same plane. However, spheroids that are located very near the edge of the well invade generally more in depth and are for

that reason excluded from the analysis. Of course it would be possible to perform the analysis in 3D, in order to get a more accurate result. But this requires sophisticated hardware and software and the benefit of calculating volume instead of using the area may not outweigh the trouble. Especially since we found that the variation in starting size of the 3-dimensional spheroids as measured in 2D, is generally only 5-10%.

Another disadvantage of this method is that the imaging is a time consuming step, since the spheroids are located at different heights, which hampers automated imaging. Also the analysis step is time consuming and requires pictures with good contrast from spheroid to matrix in order to make use of Photoshops ability to automatically recognize the border of the spheroid. In case the contrast is too low, one can easily adapt the brightness and contrast using the LEVELS and CURVES commands in Photoshop. Another way to increase the contrast would be the use a vital fluorescent dye.

As an alternative way of quantification, one could draw the area around the spheroid manually using Image J. An advance of Image J is that it is freeware (http://rsbweb.nih.gov/ij/). However, we found the manual drawing of the irregular shape of the invaded spheroids in Image J a time consuming step compared to Photoshop's Quick Selection Tool.

The protocol can be adapted to investigate invasion of other cell lines, provided that the cells are able to form spheroids.Furthermore, the optimal concentration of TGF-β to induce invasion of the cell line might be different. In all our cell lines, TGF-β induces invasion at 5 ng/ml, although M4 performs better when using 2 ng/ml.

In addition, this assay can be adapted to measure invasive responses to other growth factors, such as epithelial growth factor (EGF) or hepatocyte growth factor (HGF).

In our assays the basal and TGF-β-induced invasion was strongly inhibited by treatment with SB-431542, an inhibitor of type I receptor for TGF-β. This is a proof of principle that chemical inhibitors can be used in this assay. Other chemical inhibitors, such as MMP inhibitors, have also been used successfully at concentrations recommended by the manufacturer for monolayer cell culture.

Upon mastering this techniques, one can test the effect of chemical inhibitors, knockdown or overexpression of genes on invasion. Furthermore, by scaling up the assay to a 24 well format, one can isolate RNA using a phenol-chloroform-based method (e.g. Trizol® or Tripure) followed by a silica-column based clean-up. This RNA can be used for quantitative real-time PCR.

Our spheroid invasion assay resembles the *in vivo* process more closely than 2D invasion models, because cells in spheroids are in different metabolic states and interact in more natural fashion with their surroundings[10]. We have performed Propidium Iodide and Fluorescein DiAcetate staining to check for dead and living cells after the invasion assay and observed that similarly to the *in vivo* situation, the cells in the center are dead and necrotic, whereas the cells at the outer edge are metabolically active.

We used type I collagen rather then matrigel, because several reports have shown that the composition of extracellular matrix in breast cancer is often altered, resulting in fibrotic stiff foci with a high collagen I content. It has been demonstrated that increased collagen I content promotes breast cancer formation and invasion[15] and is associated with greater incidence of metastasis[16]. Many tumour cells thus have to invade through a collagen I rich environment in order to metastasize.

Several 3D models have been developed over the past decades. Cells can be either completely embedded within in a matrix or placed on top of a matrix or a polymeric scaffold[17,18]. In a 3D model developed by Bissell and co-workers, cells were grown in a reconstituted basement membrane (rBM) matrix. This model provides a rapid assay to distinguish between normal and malignant mammary epithelium, but focuses on cell morphology[19,20]. Morphogenesis and organization of the distinct MCF10A cell lines inversely correlated with malignancy[21,22]. In other 3D models multicellular spheroids showed the same resistance to cytotoxic drugs as their parental cell line *in vivo*, whereas cells in monolayers failed to do so[11]. Also 3D cultures of MCF10A cell lines have been used to assess sensitivity to kinase inhibitors[23]. Our spheroid model complements these assays by specifically focusing on invasion.

Disclosures

No conflicts of interest.

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References

1. Akhurst, R.J. & Derynck, R. TGF-β signaling in cancer--a double-edged sword. *Trends. Cell. Biol.* **11** (11), S44-S51 (2001).
2. Massagué, J. TGF-β in Cancer. *Cell.* **134** (2), 215 (2008).
3. Ghellal, A., *et al*. Prognostic significance of TGF-β1 and TGF-β in human breast carcinoma. *Anticancer. Res.* **20** (6B), 4413 (2000).
4. S.M., Sheen-Chen, *et al*. Serum levels of transforming growth factor-β1 in patients with breast cancer. *Arch. Surg.* **136** (8), 937 (2001).
5. ten Dijke, P. & Hill, C.S. New insights into TGF-β-Smad signaling. *Trends Biochem. Sci.* **29** (5), 265 (2004).
6. Moustakas, A. & Heldin, C.H. Non-Smad TGF-β signals. *J. Cell Sci.* **118** (Pt 16), 3573 (2005).
7. Soule, H.D., *et al*. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.* **50** (18), 6075 (1990).
8. Strickland, L.B., *et al*. Progression of premalignant MCF10AT generates heterogeneous malignant variants with characteristic histologic types and immunohistochemical markers. *Breast Cancer Res. Treat.* **64** (3), 235 (2000).

9. Santner, S.J., *et al*. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res. Treat.* **65** (2), 101 (2001).
10. Smalley, K.S., Lioni, M., & Herlyn, M. Life isn't flat: taking cancer biology to the next dimension. *In Vitro Cell Dev. Biol. Anim.* **42** (8-9), 242 (2006).
11. Kobayashi, H., *et al*. Acquired multicellular-mediated resistance to alkylating agents in cancer. *Proc. Natl. Acad. Sci. U. S. A.* **90** (8), 3294 (1993).
12. Inman, G.J., *et al*. SB-431542 is a potent and specific inhibitor of transforming growth factor-b superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol. Pharmacol.* **62**, 65 (2002).
13. Wiercinska, E., *et al*. The TGF-β/Smad pathway induces breast cancer cell invasion through the up-regulation of matrix metalloproteinase 2 and 9 in a spheroid invasion model system. *Breast Cancer Res. Treat*. **128**, 657 (2011).
14. Tang, B., *et al*. TGF-β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J. Clin. Invest.* **112**(7), 1116 (2003).
15. Provenzano, P.P., *et al*. Collagen density promotes mammary tumor initiation and progression. *BMC. Med.* **6**, 11 (2008).
16. Ramaswamy, S., *et al*. A molecular signature of metastasis in primary solid tumors. *Nat. Genet*. **33** (1), 49 (2003).
17. Debnath, J. & Brugge. J.S. Modelling glandular epithelial cancers in three-dimensional cultures. *Nat. Rev. Cancer* **5** (9), 675 (2005).
18. Pampaloni, F., Reynaud, E.G., & Stelzer, E.H. The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell. Biol.* **8** (10), 839 (2007).
19. Lee, G.Y., *et al*. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat. Methods.* **4** (4), 359 (2007).
20. Petersen, O.W., *et al*. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **89** (19), 9064 (1992).
21. Debnath, J., Muthuswamy, S.K., & Brugge J.S. Morphogenesis and oncogenesis of MCF10A mammary epithelial acini grown in three-dimensional basement membrane cultures. **30**, 256 (2011).
22. Li, Q., *et al.* p21-Activated kinase 1 coordinates aberrant cell survival and pericellular proteolysis in a three-dimensional culture model for premalignant progression of human breast cancer. **10**, 314 (2011).
23. Li, Q., Chow, A.B., & Mattingly, R.R. Three-dimensional overlay culture models of human breast cancer reveal a critical sensitivity to mitogen-activated protein kinase kinase inhibitors. *J. Pharmacol. Exp. Ther.* **332** (3), 821 (2010).

